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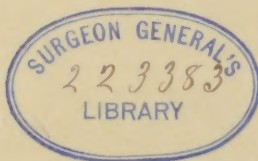
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OUTLINE OF
COMBINED COURSES IN PATHOLOGY

INCLUDING
BACTERIOLOGY AND PROTOZOOLOGY
INFECTION AND IMMUNITY
EXPERIMENTAL PATHOLOGY
HISTOPATHOLOGY AND MORBID ANATOMY

✓ BY
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COMBINED COURSES IN PATHOLOGY

PATHOLOGY AND BACTERIOLOGY

INTRODUCTION

OBJECT AND GENERAL PLAN OF THE COURSE

The scope and interrelation of the courses given in the Department of Pathology and Bacteriology will be more particularly considered in the introductory lecture of the first day. It serves as a bridge between the more fundamental medical sciences, dealing with structures and function of the body, and their application in the diagnosis and treatment of disease. In the later years of the medical curriculum, applied pathology will be utilized in the diagnosis of individual cases and in the verification of the clinical diagnosis whenever a post-mortem examination is possible. It is the object of the second-year course in pathology to present our knowledge of disease, not from the standpoint of the individual case but as a science. For this purpose the course takes up the study, first, of disease processes, and then of disease entities, beginning, so far as is possible, with the simplest diseases and extending to the more complex. Each process and disease is considered biologically from its causation, through its course, to the effect produced. Thus, in the case of infectious diseases on which we have the most complete data, we consider, first, the cause of the disease (bacteriology and protozoology), the course of the disease and the effort of the animal body to resist the disease (infection and immunity), and finally, the results produced by the disease process (histopathology and morbid anatomy). In the chronic or metabolic disease the sequence is not so clear in that causation is frequently unknown, but at least certain aspects of the course of these diseases can be studied experimentally (experimental pathology, which course might properly be called pathological physiology and chemistry). The constant correlation of the instruction given in the various courses, as listed, in the department will be evident, usually daily, and in all events weekly. It is hoped that this syllabus will aid in presenting the line of continuity and correlation in a concrete manner, as well as affording certain detailed information of a technical nature.

LIST OF REFERENCE BOOKS AND JOURNALS

Required Books

Text-book of Pathology, Adami & McCrae, published by
Lea and Febiger Price \$5.00

Manual of Bacteriology, Muir & Ritchie, 6th edition “ 3.25

In addition to the required books, the following journals and books of reference are suggested as of value in collateral reading:

*Current Journals.—**(a) Bacteriology:*

Annales de l'Institut Pasteur.

Zeitschrift für Hygiene und Infektionskrankheiten.

Centralblatt für Bakteriologie, Parasitenkunde und infektionskrankheiten; Originale; Referate.

(b) Infection and immunity:

Zeitschrift für Immunitätsforschung und experimentelle Therapie;
Originale, Referate.

Zeitschrift für Chemotherapie und verwandte Gebiete.

(c) Morbid anatomy and histopathology:

Virehows Archiv für pathologische Anatomie und Physiologie und für klinische Medizin.

Beiträge zur pathologischen Anatomie und zur allgemeinen Pathologie.

(d) The following journals cover all these fields:

The Journal of Medical Research.

The Journal of Experimental Medicine.

The Journal of Infectious Diseases.

The Journal of Pathology and Bacteriology.

The Journal of Hygiene.

Compilations.—

The following is a review of the work done in the respective fields mentioned:

Lubarsch und Ostertag, Ergebnisse der allgemeinen Pathologie.

Baumgarten's Jahresbericht über die fortschritte in d. Lehre von pathogenen Mikroorganismen.

Weichardt's Ergebnisse der Immunitätsforschung.

The Bulletin de l'Institut Pasteur is an admirable current summary of literature, particularly in bacteriology and immunity; also The Review of Bacteriology, Protozoology and General Parasitology.

Representative Books of Reference.—

(a) Bacteriology and protozoology:

Chester, A manual of determinative bacteriology.

Duclaux, Traité de Microbiologie.

Döflein, Lehrbuch der Protozoenkunde.

Hiss & Zinsser, A text-book of general bacteriology.

Jordan, Text-book of general bacteriology.

Kolle & Wassermann, Handbuch der pathogenen Mikroorganismen, second edition.

v. Prowazek, Handbuch der pathogenen Protozoen.

Park & Williams, Pathogenic bacteria and protozoa.

Craig, Parasitic amoebae of man.

Minchin, An introduction to the study of protozoa.

Baumgarten, Lehrbuch der pathogenen Mikroorganismen.

(b) Infection and Immunity:

Zinsser, Infection and Resistance.

Bolduan, Immune sera.

Bordet (Gay), Studies in Immunity.

Ehrlich (Bolduan), Studies in Immunity.

Muller, Vorlesungen über Infektion und Immunitätsforschung.

Kolle und Hestsch, Die experimentelle Bakteriologie und die Infektionskrankheiten, third edition.

Kraus und Levaditi, Handbuch der Technik und Methodik der Immunitätsforschung.

Noguchi, Serum diagnosis of syphilis.

Wright, Studies on immunization.

Metchnikoff, Immunity in infective diseases.

Emery, Immunity and specific therapy.

(c) Histopathology and morbid anatomy:

Aschoff, Pathologische Anatomie.

Adami and Nicholls, Principles of pathology.
 Delafield and Prudden, Textbook of pathology.
 Cornil et Ranvier, Manuel d'histologie pathologique.
 Stengel, A textbook of pathology.
 Wells, Chemical pathology.
 Councilman, Pathology.
 Zeigler (Warthin), General pathology.
 Grünbaum, The essentials of morbid histology.
 Kaufmann, Spezielle pathologische Anatomie.
 Virchow, Post-mortem examination (trans. by T. P. Smith).
 Cattell, Post-mortem pathology.
 Krehl and Marchand, Handbuch der allgemeinen Pathologie.
 Schmorl, die Pathologisch-histologischen Untersuchungsmethoden.
 Mallory, Principles of pathologic histology.

GENERAL LABORATORY RULES

1. Do not expose the microscope to sunlight, because the direct rays will spoil the mirror and the reflected heat will injure the lenses.

2. Seek for direct source of light when using a microscope.

3. Before putting away the microscope, carefully wipe off the cedar oil from the immersion objective, using a small piece of lens paper. Should oil have been allowed to dry on the 1/12-inch objective, clean with lens paper moistened with xylol and immediately wipe dry. Never use alcohol or any other solvent.

4. Do not put anything into the mouth, or moisten labels with tongue, while in the laboratory, owing to danger of infection.

5. Handle all cultures with great care and avoid spilling them or breaking test tubes. In case any infectious material is spilled, cover it immediately with a disinfectant (corrosive sublimate solut. 1-1000 or 3 per cent solution of lysol, which will be found in large bottles on end table).

6. Always sterilize platinum wires in the flame before using. Boil your instruments (scalpel, scissors and forceps), in a 2 per cent solution of sodium carbonate before putting them away.

7. Turn Bunsen burners low when not in actual use, in order to avoid overheating of the laboratory and wasting gas.

At the end of each exercise put away all apparatus; turn off gas, water, and electric light; clean the working table.

8. Directions for the use of the sterilizers and autoclaves will be found posted near them and should be carefully noted and followed.

9. The compartment incubator used in our laboratory is so arranged that one student receives, at the beginning of the course, two drawers in which to incubate his test-tubes and plates. He should label them with his name, and is responsible for them. In case of accident, report to the professor or assistant in charge.

10. All cultures, other than those in gelatin, are to be grown in the incubator. Cultures in gelatin are kept in a special cool incubator (temperature: 20-22° C.).

11. Test-tubes and Petri dishes should be disinfected by boiling or keeping them in a strong lysol solution for twenty-four hours before cleaning them.

12. Keep complete records of each day's work, with drawings, in a note-book.

13. Students should enter the laboratory in clean white coats. Before being sent to the laundry these coats should be sterilized in autoclave. When highly infectious material has been worked with, coats should be given to the assistants for disinfection before leaving the room.

14. Supplies can be procured from the store-room only between 2 and 3 o'clock P. M.

List of Apparatus

Each student will have issued to him certain glassware and other apparatus for which he will sign a list-receipt. He will be charged for any breakage or loss of articles on this list during the course. He will be charged also with his proportionate amount of alcohol and cotton.

The student should furnish himself with the following articles:

- White coats
- Dennison labels, Nos. 259, 256
- Debrand forceps, 1 pr.
- Coverslip forceps, 2 prs.
- Kolle needle holder
- One platinum loop
- One platinum wire
- One pair rubber gloves (wt. E, Faultless Rubber Co. best)
- One box slides
- One box cover slips, 18 x 18 mm. thickness

One 2 c.c. hypodermic syringe Sub Q, Luer, or Record
 One blue pencil for marking glassware
 One set of dissecting instruments
 One old, clean handkerchief
 One laboratory notebook

The following dyes and chemicals used, particularly in bacteriological, protozoological, and histopathological work, will be issued as needed:

Gelatine
 Peptone (siccum), Witte or Chapoteaut
 Lactose
 Mannite
 Aniline oil
 Liqueued carbolic acid
 Formaldehyde (40 per cent), in glass bottles; keep dark
 Litmus paper, blue and red
 Litmus solution (Kübel and Tiemann)
 Nutrose
 Glucose
 Glycerine
 Methyl violet (Hoechst), 6B or BN
 Fuchsin
 Eosin
 Methylene blue, medical
 Bismarck brown
 Carmine
 Picric acid
 Methyl green
 Orcein D
 Haematoxylin solution (Delafield, Ehrlich)
 Glycerine, ether mixture, Unna
 Thionin
 Phenolphthalein, 2 per cent in alcohol
 Malachite green cryst. C. P.
 C. P.
 Safranin O
 Pyronin
 Neutral red
 Methylene blue, polychromatic
 Giemsa solution for Romanowsky stain

RULES REGARDING EXPERIMENTAL ANIMALS

Animals will frequently be used by students for experimental purposes, and the following rules must be strictly followed in order to avoid dangers of infection and to insure humane treatment of the animals.

1. Each animal, or set of animals, used by a student, or group of students, should be kept in a separate cage that has been carefully labeled with the name of the owner and data concerning treatment, indicating particularly whether or not it is of infectious nature. In the latter case, care should be taken to drain the cage into a receptacle containing crude carbolic acid or similar disinfectant.

2. Students' cages must be kept in attic room over the class-room. Students must not enter the animal room on the roof unless invited to do so. The animals under experimentation will be fed and watered by an attendant.

3. All experiments of an obviously painful nature on animals must be carried out under general anesthesia unless the object of experiment would be thereby vitiated. The necessity of operations without anesthesia must be decided by the instructors.

4. At the conclusion of an experiment, except when the animals have returned to an essentially normal condition, they are to be killed by chloroform.

Observation of infected animals

5. An inoculated animal should be placed, by itself, in a suitable cage or jar; when a number of animals are injected with the same material they may be placed in the same cage. They should be numbered and designated by their color, by their curly or smooth hair, etc., and, if need be, by painting the nose, back, or flank with anilin dyes.

A complete record should be made at the time the work is done. All experimental animals should be weighed before infection and every two days afterward. Each weighing should be done, as nearly as possible, under the same conditions as the first one. The animal should have plenty of food and water before weighing. In warm weather water is imperative. A difference of 200-400 grams between two weighings may be due entirely to the withholding of water. By observing these precautions the results of weighing are of significance. It is the best index of the physical well-being of the animals. A steady, slight decrease in weight is a sure indication of a chronic, wasting disease.

The *temperature* of the animal should in many experiments be taken before beginning the experiment and subsequently on each successive day at the same hour.

The ordinary clinical thermometer, previously covered with vaseline, should be inserted well up into the rectum.

6. The post-mortem examination of infected animals is carried out on special pans which have a slightly elevated margin to prevent the blood or fluids from escaping. Rabbits and guinea pigs are bound by means of strings, whereas mice are pinned to the pans by means of long needles. The dead animals are put, after the examination has been made, in a large vessel containing crude concentrated sulphuric acid, where they soon become dissolved. (A receptacle of this type will be at hand during the entire course.)

GENERAL DIRECTIONS FOR CONDUCT OF A HUMAN AUTOPSY

An autopsy should be begun with a knowledge of all available clinical data; only with this information can it be conducted to the best advantage of both clinician and pathologist. An autopsy has for its objects the collection of data concerning the macroscopic alterations in all the organs of the body, the preservation of material for microscopic examination, which will extend the observations made on the gross material, and finally the making of such bacteriological or other examinations as shall demonstrate the cause of the morbid process or the study of intercurrent and other terminal infections. The result of the above observations should then be correlated with the functional aspects of the disease as obtained from the clinical data, and so we arrive at a picture and interpretation of morbid process as a whole.

Certain autopsies have primarily a medico-legal aspect. For the conduct of such, special procedures are necessary. Information bearing on this phase may be obtained from such books as Cattell, Letulle, etc.

Every autopsy has a legal aspect in so far as the right of performance is concerned. Laws on this subject vary from state to state, and a physician must make sure of his vested right to perform the post-mortem examination or he will be liable to civil or criminal prosecution.

A systematic procedure should be adhered to in the conduct of autopsies, but this does not preclude variations from a routine as may be indicated in special cases. The sequence in operation and note-taking as given below is advised. Never remove a part till a sufficient examination of its environment precludes the possibility of important relations being disturbed.

1. *Inspection followed by palpation, when indicated, of the body surface*

- (a) Race.
- (b) Sex.
- (c) Length of body.
- (d) Apparent age.

(e) General development of bony framework and musculature.

(f) State of general nutrition.

(g) Skin—its texture, color, discoloration due to alterations after death or to disease, decubitus, superficial appearance of wounds or operative procedures, local skin diseases.

(h) Inspection of special parts—head, including eyes (sclera and pupils); neck, including venous engorgement; chest, size and shape; breasts; abdomen (distention or retraction); external genitalia; extremities.

(i) Distortion of size or shape of parts of the body, and whether the same be symmetrically disposed.

(j) Rigor mortis—its extent and intensity.

(k) Edema of superficial parts should be accurately noted, also subcutaneous emphysema.

(l) The condition of the palpable lymph nodes.

(m) Percussion may also be used as in suspected pneumothorax, etc.

2. *Internal Examination*

The incision from which it is rarely necessary to vary for opening the abdominal and thoracic cavities extends from the suprasternal notch to the symphysis pubis; when necessary, this may be extended over one or both femoral regions for inspecting the femoral vessels for thrombi or for obtaining specimens of femoral marrow; also in special cases the incision may be carried upwards to the larynx or to the under surface of the chin.

For examination of the cranial contents the incision extends from the mastoid region directly over the vertex to a corresponding point on the opposite side. The knife should be introduced with cutting edge outwards so as to part but not cut the hair. For removal of the spinal cord the incision is carried along the middle of the back from the base of the skull to the tip of the coccyx.

As a routine, the chest and abdominal cavities are examined before the central nervous system. The anterior median incision is made down to the sternum throughout its extent, and then more superficially to avoid cutting through the peritoneum and probably opening the intestine. It should pass to the left of the umbilicus. The peritoneal cavity is then carefully opened from ensiform cartilage to symphysis pubis. An incision made from beneath separates the attachments of the recti and pyramidales close to the pubic bones. The thickness of the panniculus is to be noted. The abdominal muscles are separated from their costal attachments and the chest muscles cleanly dissected from the sternum and ribs to about 5 cm. beyond the line of costo-cartilaginous junctures. The chest muscles are incised and their color noted and the breast glands examined. The position of the diaphragm is found by a palpating hand

introduced into the abdomen. The rib cartilages, except those of the first ribs, are then cut through as near as possible to the rib juncture, with the knife held nearly horizontally to avoid incision of underlying structures. The diaphragm is then separated from the sternum, the latter elevated, and the soft parts beneath cut through to the level of the first ribs, the cartilages of which are divided as far laterally as possible. The knife then opens the sternoclavicular articulations from behind and the dissection continued for the removal of the sternum. Should the rib cartilages be calcified and the sternum brittle, it is advisable to make the dissection through the sterno-clavicular articulations and the first ribs from the front, because lifting the sternum may fracture it; in case of this latter procedure care should be taken to avoid carrying the knife too deeply or the large vessels at the root of the neck may be divided. The sternum, especially its posterior surface, is then described.

The serous cavities are then investigated—the pleural, the pericardial, and the peritoneal—sufficiently to obtain the general relations in the case and so enables one to determine the best procedure.

In the pleural cavity should be noted the degree to which the lungs retract, the degree to which the pericardium is uncovered, the sheen of the pleural surfaces, the presence of the normal amount of moisture, the presence and amount of serous transudate or of inflammatory exudate, its character, distribution, and amount; the presence and character of adhesions and their extent must be noted. Examine the thymus region. Open the pericardial cavity longitudinally, make the same observations as to contents. Note the relative size of the chambers, including the auricular appendages. In some cases, as aneurism of the arch or adjacent vessels or of sudden death possibly due to pulmonary embolism, it is advisable to make a dissection of the heart and vessels in continuity or to open the pulmonary artery before removal of the heart. In general, however, the heart is removed without previous dissection. The procedure consists of elevating the organ by slipping the left hand beneath it, cutting the vessels as closely to their point of emergence from the pericardial sac as possible; one begins from below and divides in sequence the inferior vena cava, the pulmonary veins, the superior vena cava, the pulmonary artery, and aorta. The heart is then inspected superficially, attention being directed to the amount of superficial fat, the epicardium, the surface appearance of the coronary arteries, the location, type and extent of epicardial thickenings. Measurements of external size may be made. In opening the heart, the chambers are investigated in sequence following the circulation. Hold the heart with its base toward the operator. Open the right auricle by introducing the scissors through the superior and inferior vena cavae and cutting. A second incision made nearly at right angles to the first extends across the auricle to the tip

of the auricular appendage, which should be examined for thrombi. Introduce the scissors through the tricuspid valve as far as the apex of the ventricle and cut slightly posterior to the extreme right edge; then beginning at about the middle of the anterior ventricular flap make an incision extending through the pulmonary artery. Note the character of the contained clots. The right side is then inspected, noting the size of the chambers, the thickness of the wall, the color of the musculature, infiltration with fat or connective tissue. The endocardium, and especially the valves, are then described.

The left auricle is opened by connecting the mouth of the pulmonary veins, the left auricular appendage is investigated, the interauricular septum is examined for the foramen ovale. Note the size of the cavity. The mitral valve is inspected from above. The ventricle is opened by passing the scissors through the mitral valve and cutting to the apex, the line of incision being carried as far to the left as possible. From the apex the final cut follows the left edge of the interventricular septum, passing behind the mitral valve, and through the aorta between the segments of the aortic valve. When indicated, this valve also should be inspected before sectioning it. The chambers of the heart usually contain postmortem clots, and occasionally thrombi, which are always autemortem. Their differentiation is of fundamental importance. No more concise and accurate description is found in the literature than that of Welch* who says, "These moist, pale yellowish, smooth, elastic, uniform, more or less translucent, fibrinous clots, softer or firmer according to their content of serum, non-adherent though entangled with the muscular columns and trabeculae, often showing moulds of the valves or other projecting surfaces with, at least, some red cruor clot at their most dependent parts, clots, membranous, polypoid, band-like, or filling the right cavities of the heart and sending worm-like offshoots into the vessels, should never be mistaken for the drier, opaque grey or reddish grey, granular, more friable, usually much smaller, adherent, often centrally softened or stratified thrombi." The left ventricle should then be examined for the size of the cavity, the consistency, color, translucence, friability, and thickness of the myocardium. The occurrence of fatty infiltration, of connective tissue overgrowth, and of focal inflammatory or myomalactic areas should be described. The endothelium, the papillary muscles, the chordae tendineae, and finally the valves are examined and described. That portion of the aorta which has been removed with the heart is examined for endothelial nodular or striaform thickenings, ulcerations of the endothelium and dilatations, either diffuse or focal. Section the aorta and note its thickness, and the condition of the media as well as

* Welch, in Albutt's *System of Medicine*.

the intima. The coronary arteries are then opened and followed with a fine scissors as far as possible, especially the descending branch of the anterior coronary. The organ should then be weighed and such measurements made of internal parts as may be indicated. Weight of heart, male 300 grams, female 250 grams; thickness of wall of left ventricle 1.1 to 1.4 cm.

It may be stated once for all that description of color of surfaces should be made at once on exposure, for the oxygen of the air soon makes the surface appear redder than it should, through the formation of oxyhemoglobin.

The lungs are then removed. Separate adhesions, taking care to avoid compression or tearing of the lung. When the adhesions are dense, strip the parietal pleura with the lung. Roll the organs over beyond the mid-line and remove by cutting through the bronchi close to the root of the lungs. For each lung describe the surface, color, elasticity, and volume. Compare the anterior with the more dependent portions. Note the heaviness and air content of lobes and parts of lobes. Palpate the organs using no more force than necessary and note the relative consistency of different portions, also the position, size, and consistency of deep-lying processes. Section the lung by an incision extending from apex to base along the lateral convexity and reaching well to the root of the lung. The middle lobe of the right lung should be divided by a cut along its long axis. Describe the cut surface as to color, including pigmentation, fluid content, distribution of lesions, their color, character of surface, size, and consistency, or (with destructive lesions like cavities), their size, contents, and the appearance of their walls. In tubercular cavities, septa containing vessels are not infrequent, and extension of the infection to the intima may lead to an acute miliary dissemination or to thrombosis. Such dissection of vessels and bronchi are to be made as the case may indicate. The bronchial lymph-nodes should be incised and such examination made of the remainder of the mediastinal contents as may be indicated. The thoracic aorta is opened later.

Removal of the organs of the neck is accomplished by allowing the head to fall backwards over the edge of the table and then with a long thin knife separating the skin from the larynx and neck muscles as high as the chin and along the rami of the jaws; separate the oesophagus and pharynx from the vertebral column as high as possible. Introduce the knife from below the chin and following the rami closely, separate tongue muscles and mucous membrane of the floor of the mouth. Draw down the tongue over the larynx, turn the knife with its side to the tongue and feel with the point of the knife for the juncture of soft and hard palate. Carefully incise so that the soft palate, tonsils, and adjacent mucous membranes are dissected off, first on one side, then on the other. During

this process great care must be taken not to cut the overlying skin. Then again from below complete the separation of the pharynx from the vertebral column and separate any remaining attachments of mucous membrane. The oesophagus should then be opened from behind, also the trachea and larynx, the tonsils incised longitudinally, the tongue transversely; the thyroid should be sectioned, and such additional examination of lymph nodes, nerves and vessels made as the case indicates.

In the peritoneal cavity is noted the general condition of the peritoneal surface, its sheen, its moisture, the presence of transudate or inflammatory exudate, amount of each, and the character and distribution of the latter. Note the position of the omentum. Find the origin of any acute inflammatory process, investigating especially the appendix, the gall bladder, the pelvic viscera in the female especially, and the gastro-intestinal tract. Other abnormal contents in the peritoneal cavity include stomach contents, due to post-mortem digestion and rupture, inter-abdominal hemorrhage, bloody ascitic fluid, and gas. Rarely feces, foreign matters, and the products of pregnancy are found. Note chronic adhesions, their position and character; naturally they are more frequent at the places where acute inflammatory processes are frequent. Note the relative position of the organs, and any great abnormality of size. Tumors are to be accurately localized. Examine the intestines carefully, for distention or contraction, hyperemia, intersusception, volvulus, herniae, and thickening,—then the mesentery. Note its fat contents and enlargements of contained lymph-nodes.

Removal of organs for further examination by section usually begins with the spleen. Note size, color, consistency, tenseness and any thickening of its capsule. Make an incision in its long diameter, note the trabeculae, pulp, Malpighian bodies, blood vessels, and abnormal findings as infarcts, tubercles, etc. If amyloid be suspected, apply the iodine test. Weight 150–250 grams.

It is often advisable to remove the liver at this juncture, especially since the removal gives more room. Before the removal investigate the gall-bladder and ducts for stones. In cases of malignant new growth with metastases to the liver, or a possible phlebitis extending along the portal vein as indicated by liver abscesses, the liver should not be removed till the last. In uncomplicated cases, the hand is introduced and the liver freed on the right from the diaphragm, as far as possible; then with an assistant holding the diaphragm upwards, the falciform and coronary ligaments are cut through to include the vena cava and hepatic veins. The organ is rotated forward, downward, and to the left, and the adrenal freed. Then elevating the organs divide the hepatico-duodenal ligament while carefully inspecting the cut vein, artery, and duct; then

divide the gastroseptic attachments. Inspect the surface. Note the size and consistence, measure and weigh. Inspect the surface, note the color and mottling, the thickness of the capsule in general, as well as local thickenings, nodules or general nodularity. Locate and describe superficially placed tumors. Make incisions parallel to the long axis. Describe the parenchyma, its color, translucence, the blood, and bile contents, mottling, and its relation to the lobule; note size and regularity of the lobule. Note whether the cut surface feels fatty. Examine for focal necrosis, acute inflammatory processes, carefully inspect the vessels; look for miliary tubercles when indicated. Note the presence of connective tissue overgrowth, whether it be focal or diffuse, and its relation to the lobules. Amyloid may be found. Examine for gummata, especially where there is a focal connective tissue overgrowth. Look for metastatic tumor nodules in appropriate cases. Weight, 1500-1800 grams.

Examination of the gall-bladder may be made before the liver or following it. Note its size, degree of distention, thickness of the walls, its contents, and mucous membrane.

The gastro-intestinal tract may now be removed entirely, or leaving parts in place, as special relations of lesions or the obtaining of gross specimens would indicate. In cases of obstructive jaundice the removal (coming from below) stops with the duodenum so that the bile-ducts may be opened from the liver to the papilla. Also in acute pancreatic disease the same precautions are observed. In intestinal infarcts the responsible branches of the mesenteric artery or vein are to be investigated before removal. Such additional data are obtained from the mesentery, especially the contained lymph-nodes, as is indicated. Cut off the omentum. Begin the removal of the intestines at the lower end of the sigmoid. Free it from its mesentery and carry the dissection well down behind intestine to the caput of the cecum. Examine the appendix. Thence to the small intestine, which is made taut by an assistant, the mesentery being layed over the fingers of the operator's left hand while with a sharp knife held almost parallel to the intestine, it is separated cleanly from the mesentery. The section may stop at the duodenum or by careful dissection the intestine is separated from the head of the pancreas and the stomach is then removed with the intestine. The stomach and intestine are usually opened at the sink. The incision for the stomach is parallel to and a few cubic centimeters from the lesser curvature. The small intestine is best opened along the line of mesenteric attachment, the large, along one of the muscular bands. Examine the contents of all parts of the gastrointestinal tract. Note parasites. The mucosa is inspected for its color, thickness, edema, ulcers, lymphnodes and new growths. The findings within the gut are compared with external appearances.

The pancreas is removed and sectioned, usually transversely, at several places. Note the color, consistency, and connective-tissue content. The mesentery is also removed and examined as the case indicates.

The adrenals may be removed singly or in relation to the coeliac plexus. This may readily be found by following the splanchnic nerves to the plexus, carefully freeing it, and lifting the adrenals in connection with the plexus. The adrenals are cut transversely and inspected. Tuberculosis, thrombosis of the central vein, and small or large tumors may be found.

The urinary tract may be taken out as individual organs or as a whole. Where there is no special indication for the latter procedure, the kidneys are freed by an incision lateral to the organ in the perirenal fat. Free the organs by dissecting with the fingers, inspect each superficially, and remove by cutting through the vessels and ureter. Weigh. Incise longitudinally. Strip back a part of the capsule, note the size, surface-markings and vessels, color, translucence, and consistency, describe inflammatory foci as abscesses, tubercles, etc. On the cut surface note the relative proportion of cortex, pyramids, the thickness of each, and distinctness with which they are differentiated. Then investigate the general architecture of the cortex, including glomeruli—the medulla, and the vessels, the presence and distribution of connective tissue infiltration. Again note inflammatory foci. Examine the vessels at the hilum. Finally examine the pelvis. Weight of kidneys, together 300 grams, the left kidney about 5 grams the heavier.

The pelvic viscera are most easily removed *en masse*. Separate the peritoneum about the brim of the pelvis, beginning anteriorly. Most of the dissection can be made with the fingers, separating the pelvic viscera completely from the wall of the pelvis, extending the dissection well down to behind the rectum. With traction from above, the posterior urethra, prostate and rectum are successively cut through and finally the vessels, spermatic cords and ureters. The rectum is then examined. The bladder is opened at the vertex and the incision carried longitudinally. The mucosa is examined, especially that of the trigonal region. The incision is carried through into the urethra along its dorsal mid-line and its prostatic portion observed. Incise the prostate transversely. In pulmonary or right-sided cardiac embolism, examine the pelvic veins for thrombi. Dissect the rectum from the bladder and examine the seminal vesicles. The testes are obtained for examination by making a horizontal slit beneath the skin just lateral to the symphysis on either side and then evaginating the organs. Examine the epididymes and testes, the latter by a longitudinal incision. Note size and evidence of connective tissue overgrowth. In case of tuberculosis, the epididymis is often affected and in such cases the genito-urinary tract may be

removed entire so as to demonstrate anatomical relations of the process. Syphilis attacks the testis. In the female the internal genitalia are examined as follows: The uterus—its size, position, adhesions, and tumors. Incise longitudinally in its anterior mid-line and then make oblique incisions to open the cornua. The ovaries are sectioned longitudinally; the tubes are also to be examined. The vagina is best incised on the side.

There still remain for examination the aorta and the vena cava, the thoracic ducts, the retroperitoneal lymphatics, and the sympathetic ganglia. Open the aorta in situ with scissors; then remove, note and describe, elasticity, dilatations, the intima and its lesions, finally the media. The thoracic duct lies behind the aorta and slightly to the right. The azygos vein lies to the right of it. The receptaculum chyli lies on the second or third lumbar vertebra. Its examination is important in tuberculosis, which apparently originates in the intestines and mesenteric lymph-nodes.

The femoral bone marrow is obtained by extending the mid-line incision over one of the femora, making two saw cuts about 5 cm. apart, and chiseling off the intervening bone. Be careful not to fracture the femur.

In examination of the central nervous system the scalp incision is made as noted above. The scalp is reflected anteriorly to within 2 cm. of the opening of the orbits. The posterior flap is reflected so as to well uncover the occipital protuberance. This dissection is carried on in such a way as to completely remove all connective tissues from the bone. The calvarium is inspected superficially. The temporal muscles are incised in the line of the subsequent saw cut, and its divisions reflected upwards. The incision in the calvarium should begin about 2.5 cm. above the orbits and be carried circularly around the calvarium to just beneath the protuberance. This method gives the best access to the brain, but it is often advisable to make a wedge-shaped incision which is less likely to be followed by the skull-cap slipping out of place after the body is restored. The latter procedure consists in making a circular incision extending over the frontal region down either side to slightly behind the ear, and from the latter points carry lines which meet just in front of the protuberance. Do not saw too deeply, especially in the relatively thin temporo-parietal regions. After the outer table has been well divided, insert a chisel anteriorly and with sharp blows break through the inner table; repeat the process, especially at the angles of the incision and posteriorly. When the calvarium is loose, insert a hook anteriorly and with a sharp pull the calvarium is generally easily removed, and the underlying dura should be quite intact. When fracture of the skull from trauma is a possibility, the bone must be

completely sawn through. In certain cases, for example young children and the aged, the adhesion between the skull and dura does not allow the the above method of removal of the calvarium and it is necessary to divide the dura with a scissors along the line of the saw cut.

Examine the skull on removal. Note its thickness, texture, the relative amount of diploe, local thickenings, or thinnings, and local depressions, and fractures. Examine the dura; open the longitudinal sinus with blunt-pointed scissors. Open the dura by an incision parallel to and about 5 cm. above the line of saw cut in the skull; separate the falx anteriorly, being careful not to cut the brain tissue. Examine the visceral surface of the dura; note evidences of acute inflammatory changes, false membranes so-called, and internal hemorrhages. At the same time examine the underlying brain as far as exposed. Remove the brain as follows: Elevate the frontal lobes, carefully separate the olfactory bulbs, if necessary. Then, using a long thin knife, divide the optic nerves just behind the foramina, next the third and fourth nerves and the infundibulum, followed by the carotid arteries. Now rotate the left hemisphere so as to expose the tentorium and inserting the knife, with cutting edge outward, separate the tentorium from the petrous portion of the temporal bone. Repeat on the opposite side. Then section the cranial nerves in sequence on both sides and finally, inserting the knife as far as possible through the foramen magnum, cut through the cord and the vertebral arteries. The brain is then easily rolled backwards on to the supporting left hand with the assistance of the right introduced and making slight traction from below the cerebellum. Note the amount of cerebro-spinal fluid. Examine the brain. Note the symmetry, its color, its consistency in general, together with local variations. Examine the convolutions. Are they normally disposed? Note flattening or narrowing of convolutions. Describe the pia, its thickness, the amount and position of cerebro-spinal fluid, inflammatory manifestations and their distribution. Describe the vessels at the base. Note atrophies, focal lesions, and tumors, with complete description of location and special characteristics. Examine the floor of the fourth ventricle for granulations. Such blocks of tissue as are wanted for special fixation should now be taken from the brain and the remainder preserved intact in 10 per cent formalin in normal salt solution. Except in medico-legal cases, general dissection of the brain is neither necessary nor desirable. Clinical curiosity had much better be held in check for two weeks while the brain is becoming thoroughly hardened in the formalin, after which all dissections may be made to much greater advantage than at an autopsy. Now examine the dura over the base of the cranial cavity, and open the sinuses as may be indicated. The hypophysis is readily removed by fracturing with a chisel the clinoid process transversely at a level with

the base of the sella turcica; pull the bone fragment backward and grasping the dura with forceps shell out the gland. Removal of special parts, as the retina, the internal ear, and inspection of the naso-pharynx may be readily accomplished by special methods which may be found described and figured in many books. Mallory & Wright's *Pathological Technique* is particularly to be recommended.

Removal of the spinal cord is accomplished by dissecting the muscles from the vertebrae as fully as possible from the nape of the neck to the coccyx. Then with a two-bladed saw (double rachitome), the blades being adjusted to just clear the widest of the spinous processes, saw just through the transverse processes, from neck to about third sacral vertebra; from this level with a single saw converge the lines of incision to meet over the coccyx. It may be necessary to loosen some of the transverse process in the lower lumbar region with a cleaver chisel. Then lift off the separated bony portions from below upwards. In the cervical region it is often necessary to cut through the upper transverse processes with a chisel. When it is necessary to remove the posterior root ganglia, these are freed by using a chisel or strong bone-forceps for breaking the bony parts, and a long thin knife to separate the nerves. The cord is removed by firmly seizing the dura near its lower extremity, preferably with a mouse-tooth clamp-forceps and dividing the nerves as far outward as possible. Continue this, using little traction and less bending till the whole cord is removed encased in the dura. Lay the cord flat and open the dura posteriorly throughout its extent. Describe the dura, pia, and cord as a whole. Make, with a very sharp knife, only such transverse sections as are necessary. Describe the appearance of the cross-sections. Remove sections for special fixation and place the cord with the brain in 10 per cent formalin in salt solution, keeping the segments as straight as possible.

As the various organs are being examined, features are presented which call for histological study or which make it desirable to preserve the specimen as a whole. In the latter case care should be taken to make only those incisions which are necessary and which do not mar the ultimate purpose as a specimen. Color preservation as well as preservation of form is usually desirable in a gross specimen. The best method for this purpose is that of Kaiserling, which consists in placing the specimen, after necessary dissection and arrangement, in the following solution

Potassium acetate	30 gms.
Potassium nitrate	15 gms.
Formaldehyde (40 per cent)	200 c.c.
Water	1000 c.c.

for sufficient time to insure penetration. The process should be carried on in the dark. The color which is altered by the above solution is restored by immersion in 80 per cent followed by 95 per cent alcohol, the time again depending on the size of the specimen. Twelve to twenty-four hours in each is often sufficient. The specimen is then passed into either Kaiserling's third fluid

Potassium acetate	200 gms.
Glycerine	400 c.c.
Water	2000 c.c.

in which it is mounted, or into pure glycerine for twenty-four hours or more, followed by paraffin oil (Wilson's mounting medium), for sufficient time to remove the glycerine; it is then mounted in fresh oil.

3. *Fixation*

For the preservation (fixation) of tissues for microscopic examination, a great variety of methods are available and the end in view should determine the method. Use plenty of fixing fluid. In all cases the blocks of tissue should be sufficiently thin to allow speedy penetration. They should be cut with a sharp knife from unmanipulated tissues, not from areas which have been pinched, washed in tap water, or allowed to dry in the air. The earlier after death the tissue is secured, the more satisfactory will be the results.

Alcohol (95 per cent) is useful where bacteria or fibrin are to be stained, and for the gray substance of the central nervous system. *Absolute alcohol* should be used for the micro-chemical demonstration of glycogen. In general, fix tissues not over 5 mm. thick in 95 per cent alcohol. Change the alcohol in a few hours and again after twenty-four hours. After forty-eight hours, if the tissue is not to be embedded immediately, it is preserved in 80 per cent alcohol. Nerve tissue, however, is kept in 95 per cent alcohol.

Formaldehyde.—4 per cent solution in normal salt solution (i.e., 10 per cent of the commercial solution of formaldehyde, which should contain 40 per cent of the gas), is especially useful where frozen sections are to be made, also where it is necessary to take large blocks of tissue on account of the rapid penetration by the formaldehyde. Again for the same reason it is extensively used to preserve the central nervous system after such tissue-blocks as may be wanted for special methods have been removed. The brain is completely fixed in two weeks and may be sectioned in any plane without the great distortion seen when the brain has been slashed at the autopsy. After its use, also, the tissue may be mordanted in a number of ways which makes special staining methods

available. The most frequent example of such mordanting is in Muller's fluid and then staining the myeline sheaths by Weigert's method (see below). Tissues may be left almost indefinitely in formaldehyde solution but gradually lose their finer staining qualities. From the formaldehyde solution blocks of tissue are transferred to 95 per cent alcohol for dehydration preliminary to imbedding, unless they are to be specially mordanted before dehydration.

*Muller's fluid*¹ is used today almost exclusively for the central nervous system, either alone or following formaldehyde as indicated above. By its direct use more brilliant result may be obtained by the Weigert myeline method, than after the preliminary formaldehyde fixation. The same holds true for the Marchi-Algeri method for demonstration of oleic fat.

Orth's fluid is 4 per cent formaldehyde in Muller's fluid. It is a useful fixative for organs containing much blood, for bone marrow, and bone, and is not without merit as a general fixative. Fix tissue 0.5 to 1 cm. thick up to four days, changing the fluid after twenty-four hours. Wash in water twenty-four hours and place tissue in 80 per cent alcohol.

Zenker's fluid (2) is the best fixative for general use. After it a variety of special stains are applicable as well as the usual ones. Eosin and methylene blue staining gives particularly useful results; phosphotungstic acid hematoxylin gives its best results after its use, although formaline-fixed tissues mordanted with Zenker's fluid may also be employed. Blocks of tissue rarely over 5 mm. thick are fixed for twenty-four hours, rarely forty-eight, in this solution. Wash in running water for twenty-four hours and then put in 80 per cent alcohol.

Other useful and often essential fixing methods include mercuric chloride in saturated solution in water, either alone or with the addition of acetic acid, 5 per cent, or saturated solution in normal salt solution, as is used in Wright's method for bone marrow. Flemming's fluid, which contains osmic acid, is useful for demonstration of fat—and also as a general fixative. Tissue blocks not over 2 mm. thick are fixed for twenty-four to forty-eight hours and then washed and transferred to 80 per cent alcohol.

4. Bacteriological examination at autopsies

The bacteriological examination of blood is carried out with heart blood. It is collected before any other organs have been removed from

¹ Muller's fluid: Potassium bichromate, 2.5 gms.; Potassium sulphate, 1.0 gm.; water, 100.0 c.c.

² Zenker's fluid: Potassium bichromate, 2.5 gms.; Mercuric chloride, 5.0 gms.; water, 100.0 c.c. Add 5 per cent glacial acetic acid just before using.

the body. In opening the thorax avoid injuring the large veins. The pericardium is exposed; the anterior portion of the right ventricle is sterilized by searing it with a thermocautery or a hot knife; puncture the ventricle with a sterilized glass pipette. Without difficulty, several cubic centimeters of blood can be aspirated. Various amounts of the heart blood are plated in agar or mixed with broth or bile. The latter can, with advantage, be plated out after an incubation of twelve hours. The examination is completed by the preparation of anaerobic cultures, particularly in cases of puerperal sepsis, ulcerating tumors, phlegmonous processes of the intestines, or urinary bladder.

Aside from the blood, various organs, secretions, and exudates may be the object of an examination. When cultures are desired of peritoneal, pleural, or pericardial exudate, open the peritoneum or pleura with a hot sterile knife and collect the fluids by means of pipettes.

The organs to be examined bacteriologically are carefully handled and not unnecessarily soiled with intestinal contents, etc. The surface of each organ is sterilized by searing with a hot instrument. Then an incision is made with a sterile scalpel. From the cut surface the material to be examined is removed with a platinum needle or spatulum.

The nervous tissues of the brain are cultured by searing (after removal of the dura) both hemispheres; puncture the gray and white matter with sterile cork-borers and smear the worm-like pieces on the surface of various culture media.

The cerebro-spinal is removed from the infundibulum or by lumbar puncture. For serologic tests the blood of the femoral veins is best used. It is centrifuged immediately after collection.

5. *Dehydration and imbedding of tissues*

After fixation the tissues are, as a rule, preserved in 80 per cent alcohol. Since, however, the tissues usually contain a large quantity of water when placed in the alcohol, use a large quantity of alcohol and change every twenty-four hours.

Imbedding is a process by which the blocks of tissues are infiltrated with paraffin, cellidin, or other substances which give uniform consistency and so enable one to cut sections of uniform thickness and to handle the tissue more expeditiously.

1. Cut blocks of tissue required for microscopic examination. A block up to about 18 mm. square and 2-3 mm. thick is advisable.

2. 95 per cent alcohol twenty-four hours.

3. Use absolute alcohol 8-10 hours.

4. Absolute alcohol over night.

5. Absolute alcohol and xylol, equal parts, one hour.

6. Xylol, one hour (make sure the tissue is perfectly cleared).
7. Xylol and paraffin, equal parts, one hour.
8. Paraffin (which has been used only once), one hour.
9. Paraffin (fresh), one hour.

The paraffin-xylol mixture is kept fluid on top of the imbedding oven, while the paraffin is melted, poured into suitable pans, allowed to cool to 56 degrees (use thermometer), and at 56 degrees and kept at 56 degrees in the oven. The pans must be clean. Just before using, smear the inside of the pan with a *very* thin coat of glycerin. This keeps the paraffin from sticking. After the last paraffin, arrange the blocks in the pan and carefully float the pan on the surface of ice-cold water. This insures rapid solidification of the paraffin without crystallization and gives better cutting qualities to the block.

Trim off excess paraffin, leaving a rectilinear block of paraffin, and mount on small wooden blocks, heating the latter until the surface is slightly charred, and quickly apply the paraffin block. Be sure the surface of the paraffin block is flat. Pass a hot needle around the edge of the paraffin to fill in the space between the edge of the paraffin and the wood, thus giving better support and preventing the specimen's pulling loose when sectioning.

The method for celloidin imbedding may be found in Mallory & Wright's, *Pathological Technique*. It is useful for special tissue which contain hard substances, or very thick specimens, but is not recommended as a routine.

6. Section Cutting

The microtome will be demonstrated, also the method of sharpening knives. Be sure these are understood. Be sure the knife is sharp and free from tiny nicks. Set the block with the tissue in the holder with the long diameter of the specimen at right angles with the knife. Trim off excessive paraffin.

Mounting.—The cut sections are floated on distilled water with the glossy side down. Slowly heat the dish containing the sections until the latter become perfectly flat; be careful not to melt the paraffin. Slides must be perfectly clean. Sections can readily be cut 6 micra or thinner. When tissue has been fixed in a solution containing chrome salts or osmic acid, it is necessary to use some substance which will glue the sections to the slide or they will float off in subsequent manipulations. The best for general use is a mixture of filtered egg-albumen and glycerin, equal parts of each, preserved by the addition of 1 per cent sodium salicylate (Mayer's glycerin-albumen). Place a small fraction of drop on the slide and with a clean finger rub it uniformly over the surface. Avoid excess. After alcohol, formaldehyde, or mercuric bi-

chloride fixation, it is not necessary to use the albumen-glycerin mixture. The sections being flattened, place the slide beneath one and draw it out of the water, orient the section, drain off excessive water, and allow to dry over night at room temperature or for several hours in thermostat at 37 degrees.

Frozen sections are valuable for quick diagnosis, and because of the simplicity of technic may be used for many purposes. They are essential where substances like fat are to be stained, which would be totally removed by the alcohol in the paraffin imbedding process. Liquid carbon dioxide is used for freezing. The Bardeen type of freezing microtome is used.

Prepare a block of tissue about 3 mm. thick, as large as desired up to the capacity of the plate of the freezing microtome. Make good contact between the specimen and plate, using a drop of water if necessary. Turn on the carbon dioxide slowly at first and gradually increase the flow. As soon as the surface appears frosted, turn off the gas. Hold the knife, firmly in the right hand. Lubricate each of the glass ways with a drop of water. Try the consistency of the specimen with the knife; if too hard, wait a moment; when the proper consistency is secured, by a rapid back and forth movement of the chisel held quite firmly on the glass runners, cut sections while the left hand is turning the elevating screw at a uniform rate. Such sections should not exceed 10 micra in thickness. When a small pile of sections is secured on the knife, put the knife with sections in distilled water or normal salt solution; rapidly remove water from knife and proceed with the cutting.

The sections obtained are taken up on a clean slide, carefully flattened, the excess of water removed (this is facilitated by blowing on the specimen). A thin strip of tissue paper may then be laid on the section and 95 per cent alcohol dropped rapidly to thoroughly soak the paper. Carefully lift off the paper and continue the alcohol for several drops, then apply absolute alcohol by dropping, and after complete dehydration drop on a very thin solution of celloidin. Allow this to thicken in the air (do not blow on it), but before it becomes dry hold the slide face downward on the surface of distilled water; then after a few seconds the slide may be turned over in the water, and after the alcohol and ether are removed, proceed to stain by the ordinary methods.

Staining.—The most generally useful method for staining tissue is that of hematoxylin and eosin. The hematoxylin stains the nuclei and to a greater or less extent certain basophilic substances, as the cytoplasm of plasma cells, mucous, cartilage, etc. Of the various solutions of hematoxylin, Delafield's or Ehrlich's acid hematoxylin are the most useful. In almost all hematoxylin solutions the staining property is due to the hematin contents, which is formed from hematoxylin during

the process of oxidation or ripening of the solution. It is necessary also to use some mordant, as alum or chrome salts, to cause union between the tissue and the dye. The mordant may be used separately or be contained in the hematoxylin solution. The eosin is used in simple aqueous solution; the yellowish water-soluble eosin (Grubler) is the most satisfactory for general use; it is used in about 0.05 per cent solution.

7. Procedure for paraffin sections mounted on the slide

Use Coplin jars for reagents except absolute alcohol, which should be kept in a drop bottle.

1. Remove paraffin from sections with xylol-5 minutes.
2. Remove xylol with 95 per cent alcohol-5 minutes.
3. Change alcohol.
4. Place in distilled water. Always use *distilled* water before hematoxylin.

5. Stain in 5-10 per cent solution of Delafield's hematoxylin or in Ehrlich's hematoxylin. Follow the staining under the microscope and when nuclei are distinct, stop the staining.

6. Wash thoroughly in *tap* water.

7. Again examine with the microscope and if over-stained the section may be decolorized by use of Orth's discharging fluid (1 per cent hydrochloric acid in 70 per cent alcohol), for a few seconds, followed by thorough washing in *tap* water, at least until the blue color completely returns. If permanent preparation is desired it is advisable to wash for several hours.

8. Place in eosin solution till the desired shade is attained. Follow with microscope. After Zenker solution, the eosin is readily taken up and relatively firmly retained. Wash in water if necessary to remove excess of the eosin.

9. Wipe off excess of water from slide; put in 95 per cent alcohol.

10. Clear in originum oil or drop absolute alcohol on specimen until completely dehydrated and clear in xylol. If one of the essential oils, as originum, has been used, remove it with xylol and mount in Canada balsam dissolved in xylol.

For special staining methods, such as may be required in specially assigned work, consult Mallory & Wright.

FIRST WEEK

GENERAL OUTLINE

Following is an outline of the ground covered in the combined courses, according to days, followed by more specific directions and technical detail.

Introductory lecture explaining the purpose and correlation of the courses in pathology and bacteriology.

A. Bacteriology

First Day.—Assignment of places; preparation of solutions; methods of cleaning and sterilizing glassware; use of the microscope, particularly as regards artificial illumination, and use of the oil-immersion lens.

Exercise 1.—Study of an anthrax infection.

Second Day.—Preparation of staining solutions (continued).

Third and Fourth Days.—Simple method of recognizing and isolating bacteria (continued).

Exercise 1.—Anthrax (continued).

B. Infection and Immunity

Two lectures will be given during the week on the general aspects of infection and immunity.

C. Experimental Pathology

The work in this section and considerable of the work in infection and immunity is elective, or participated in by only a few members of the class. The results will be demonstrated when practicable to the entire class.

Experiments are begun or completed which are designed to illustrate: Neuropathic atrophy; cloudy swelling; fatty degeneration; obstructive jaundice; hematogenous jaundice; calcification, pigmentation and necrosis.

D. Histopathology and Morbid Anatomy

For work in the postmortem room, sections of the class will go to the Alameda County Infirmary on Tuesday and Friday mornings of each week when material is available. The students will, so far as possible, conduct the autopsies under direction of the instructor. Each student should provide an apron (light rubber is best), and rubber gloves; pure

gum and heavy are advisable since these stand sterilization by boiling most successfully. Each group of students is responsible for the careful restoring of the body and cleaning of all instruments and of the autopsy room, unless this can be otherwise provided for. Each student will read the autopsy protocols which will be furnished by the instructor in charge during the early part of the course; later the students shall furnish them. The gross anatomical lesions will be demonstrated to the whole class and such sections for microscopic examination shall be prepared as the instructor shall indicate. The bacteriological material will also be worked up by the students.

The class work for the week consists in study of gross and microscopical preparations of normal tissues, tissues illustrating atrophies, necroses, pigmentation, calcifications and concretions.

First Day

A. BACTERIOLOGY: TECHNICAL DETAILS

1. Solutions

Solutions employed in the course will be made for the most part by the student from time to time as directed.

1. Saturated alcoholic solution of fuchsin.
2. Saturated alcoholic solution of methyl violet 6B or BN.
3. Saturated alcoholic solution of methylene blue.

For each of these solutions take twenty grams (30 grams for fuchsin) of the dye and mix it with 300 c.c. of 96 per cent alcohol and shake the solution several times during the day.

4. Bismarck brown 0.4 grams is dissolved in 200 c.c. of distilled boiling water and then filtered.

5. Safranin, 6 grams, is dissolved in 200 c.c. boiling water and when cool, filtered.

6. Lithium carmin for staining sections: 3 grams of lithium carbonate is dissolved in 300 cc. of boiling distilled water. When cold, the solution is filtered and 7.5 grams carmine is added. After a certain time filter the solution again.

7. Saturated watery solution of neutral red. Dissolve about 1 gram in 100 cc. boiling water and sterilize the solution in a drop bottle.

8. Prepare three litres of 60 per cent alcohol from 96 per cent alcohol, which means one litre of 90 per cent alcohol and 536.5 c.c. distilled water.

9. Add to one litre of 60 per cent alcohol 1 per cent hydrochloric acid.

10. One litre of 60 per cent alcohol containing 3 per cent hydrochloric acid.

11. Absolute alcohol. The absolute commercial alcohol contains always $\frac{1}{2}$ to 1 per cent water.

This may be eliminated by adding dehydrated copper sulphate (white powder). This copper sulphate is prepared by burning ordinary copper sulphate in a porcelain dish until it is white. Cover the bottom of a powder bottle with this material and add the absolute alcohol. The alcohol is filtered before use.

12. Iodine solution: Iodine is relatively insoluble in water, but very easily soluble in saturated potassium iodide solution. Dissolve in a bottle 1 gram of iodine, 2 grams of potassium iodide and 5 c.c. of distilled water. When dissolved, add another 300 c.c. of water.

13. Eosin solution. Dissolve 1 gram of eosin in 100 c.c. of water.

14. Physiological salt solution. Dissolve 8.5 grams of absolutely pure sodium chloride in one litre of distilled water. Shake, sterilize, or add a small crystal of thymol. For certain purposes, 0.5 per cent carbolic acid (pure, Merck) may be added.

15. Saturated mercury bichloride solution: 21 grams of mercury bichloride are dissolved in 300 c.c. boiling distilled water. A small quantity of the chemical will crystallize out when the solution becomes cold.

16. One litre of 2 per mil of bichloride of mercury solution for disinfection.

17. Lysol solution. A 5 per cent solution is used in a jar to disinfect the cover slips and slides.

18. 50 per cent solution of potassium hydroxide: Dissolve 100 grams potassium hydroxide in 200 c.c. distilled water and keep in a bottle with rubber stopper.

2. Cleaning and sterilizing Glassware

All glassware, used in bacteriological work should be thoroughly cleaned.

To clean new test tubes.—

1. Place the tubes in a bucket or other convenient receptacle, fill with water and add a handful of sodium carbonate soap powder. See that the tubes are full and submerged.

2. Boil the bucket over a large Bunsen flame or in the autoclave for thirty minutes.

3. Cleanse the interior of the tubes with test-tube brushes, and rinse thoroughly in cold water or in 1 per cent hydrochloric acid.

4. Invert the tubes and allow them to drain completely.

5. Dry the tubes and polish the glass inside and out with a soft cloth.

New flasks, plates, and capsules must be cleaned in a similar manner. Fill each flask with water to which some soap powder and a few crystals of potassium permanganate have been added, and let boil over the naked flame. The interior of the flask can then usually be perfectly cleaned with the aid of a flask brush, but in some cases water acidulated with 5 per cent nitric acid, or a large wad of wet cotton-wool previously rolled in silver sand, must be shaken around the interior of the flask, after which rinse thoroughly with clean water, dry and polish.

To Clean New Graduated Pipettes.—

1. Place the pipettes in a convenient receptacle, filled with water to which soap powder has been added.

2. Boil the water vigorously for twenty minutes over a Bunsen flame.

3. Rinse the pipettes in running water and drain.

4. Run distilled water through the pipettes and drain.

5. Run rectified spirits through the pipettes and drain as completely as possible.

6. Place the pipettes in the hot-air sterilizer, close the door, open the ventilating slide, and run the temperature slowly up to about 80 degrees C. Turn off the gas and allow the oven to cool; or attach each pipette in turn to the rubber tube of the foot bellows, or blowpipe air-blast, and blow air through the pipette until the interior is dry.

Glassware that has already been used is often *infected*, and is treated in a slightly different manner.

Microscopical Slides.—

The slides in general use measure 3 inches by 1 inch, or 76 by 26 mm., and should be of good white crown glass, with round edges.

New Slides should be allowed to remain in alcohol containing 5 per cent hydrochloric acid for some hours, rinsed with running water, thoroughly drained on a towel, dried, and finally polished with old linen.

If only a few slides are required for immediate use, a good plan is to rub the surface with jeweler's emery paper (Hubert's 00). A piece of hard wood 76 by 26 mm. with a piece of this emery paper gummed tightly around it is an exceedingly useful article on the microscope bench.

Cover-Slips.—The most useful sizes are the 19 mm. square for ordinary cover-glass film preparations, and 38 by 19 mm. rectangles for blood films and serial sections; both varieties must be of "No. 1" thickness, which varies between 0.15 and 0.22 mm., that they may be available for use with the high-power immersion lenses.

Cover-slips should be cleaned in the following manner:

1. Drop the cover-slips one by one into an enameled iron pot or tall glass beaker, containing a 10 per cent solution of chromic acid (see formula, footnote 1).

2. Heat over a Bunsen flame and allow the acid to boil gently for twenty minutes. A few pieces of pipe-clay or pumice may be placed in the beaker to prevent the "spurting" of the chromic acid.

3. Turn the cover-slips out into a flat glass dish and wash in running water under the tap until all trace of yellow color has disappeared. During the washing keep the cover-slips in motion by imparting a rotary movement to the dish.

4. Wash in distilled water in a similar manner.

5. Wash in rectified spirit.

6. Transfer the cover-slips, by means of a pair of clean forceps, previously heated in the Bunsen flame to destroy any trace of grease, to a small beaker of absolute alcohol.

Drain off the alcohol and transfer the cover-slips, by means of the forceps, to a wide-mouthed glass pot, containing absolute alcohol, in which they are to be stored, and stopper tightly.

They can easily be dried with a soft linen cloth and are ready for use. For certain purposes, as indicated in the exercises, it is advisable to heat the slides and cover-slips at a temperature of 150 to 200 degrees C in a hot-air sterilizer. By this process all the fats will be sublimated.

To Clean Used Culture Tubes, Petri Dishes, etc.—

Infected Test-Tubes:

1. Pack the tubes in a wire basket in the autoclave in the vertical position.

2. Disinfect completely by exposing them to a temperature of 120° C for twenty minutes (see page 37).

(If an autoclave is not available, the tubes must be placed in a digester or even a large pan or pail with a tightly-fitting cover, and boiled vigorously for, from thirty to forty-five minutes to insure disinfection.)

3. Empty each tube in turn while it is still hot and roughly clean its interior with a stiff test-tube brush.

4. Place the tubes in a bucket or other convenient receptacle; fill with water and add a handful of sodium carbonate soap powder. See that the tubes are full and submerged.

¹ Cleansing solution: Potassium bichromate, 40 grams; water, cold, 750 c.c. When completely dissolved, add slowly and carefully sulphuric acid, 230 c.c. The solution may be used repeatedly until it becomes dark colored. Dilute well with water before discarding.

5. Place the bucket over a large Bunsen flame and boil for thirty minutes.

6. Cleanse the interior of the tubes with the aid of test-tube brushes, and rinse thoroughly in cold water.

7. Drain off the water and immerse tubes in a large jar containing water acidulated with 2 to 5 per cent hydrochloric acid. Allow them to remain there for about fifteen minutes.

8. Remove from the acid jar, drain, rinse thoroughly in running water, then with distilled water.

9. Invert the tubes and allow them to drain completely. Dry the tubes and polish the glass inside and out with a soft cloth, such as old linen.

Infected Flasks, Plates and Capsules must be treated in a similar manner. **Flasks which have been used in the preparation of media must be cleaned immediately when finished with.**

Plugging Test-Tubes and Flasks.—

Before sterilization all test-tubes and flasks must be carefully plugged with cotton-wool, and for this purpose best absorbent cotton-wool (preferably that put up in cylindrical one-pound packets and interleaved with tissue paper— known as surgeons' wool) should be employed.

1. For a test-tube or a small flask, tear a strip of cotton-wool some 10 cm. long by 2 cm. wide from the roll.

2. Turn in the ends neatly and roll the strip of wool lightly between the thumb and fingers of both hands to form a long cylinder.

3. Double this at the center and introduce the rounded end into the open mouth of the tube or flask.

4. Supporting the wool between the thumb and fingers of the right hand, rotate the test-tube between those of the left, and gradually screw the plug of wool into its mouth for a distance of about 2.5 cm., leaving about the same length of wool projecting.

The plug must be firm and fit the tube or flask fairly tightly, sufficiently tight in fact to bear the weight of the glass plus the amount of medium the vessel is intended to contain, but not so tightly as to prevent it from being easily removed by a screwing motion when grasped between the fourth, or third and fourth fingers and the palm of the hand.

For a large flask a similar but larger strip of wool must be taken; the method of making and inserting the plug is identical. They are sterilized in the hot air sterilizer for one hour at 150 degrees. The Petri dishes are wrapped in paper and sterilized by hot air, or by steam. The latter is particularly advisable when thin glass is to be sterilized. For this process an autoclave with 5 kg. pressure (15 pounds), at

120° C must be used. When sterilizing glassware in the hot-air sterilizer, always let the oven cool down with closed doors before the glassware is removed from the shelves; otherwise there may be considerable breakage.

3. *Preparation of pipettes*

Glass tubing having a diameter of about 8 m.m. is cut up into lengths of about 35 cm. A slight constriction is made at a distance of about 6 cm. from each end. This is not necessary, but it serves to prevent the cotton plug from descending. Moreover, the tube can be readily sealed at this place. The ends are rounded in the blast lamp. A piece of cotton is pushed into each end of the tube by means of a drawn-out piece of glass tubing. The tubes, thus equipped, are placed in a horizontal position in a dry-heat sterilizer. The sterilized tubes should be kept in stock and from these the pipettes can be made in a few minutes, whenever desired. To make the pipette proper, the plugged sterile tube is heated at the middle in a blast lamp. The broad flame should be used in order to soften as long a piece of tubing as possible (3 to 5 cm.). When the middle is thoroughly softened, the tube is removed from the flame and the two halves should be *slowly* drawn apart. A relatively wide, thick-walled capillary is thus obtained, whereas if the tube is drawn out rapidly the resulting capillary will be very narrow and thin-walled. The drawn-out portion should be about 40 cm. long. This is then heated in the middle and, as a result, two sealed pipettes are obtained. These tubes are frequently used for the sterile collection of blood.

4. *Artificial Illumination*

Several microscopic preparations are given to practise focusing by artificial light. The artificial light used in the laboratory is electric light of 16-candle power with opaque bulb.

Try by adjusting the mirror to obtain the greatest possible amount of light; then focus the preparation with the immersion lense. Move the mirror in different directions and the Abbe's condenser up and down until a correct picture of the illuminating part, for example, the filament of the electric light, appears. By moving the Abbe a little up towards the microscope stage, the most suitable diffuse light over the entire preparation will be obtained.

5. *Material to be prepared for the next day*

1. Manure, feces, etc.

2. Put in several glasses with water, a small amount of hay and pieces of raw potatoes and incubate them at 37° C until the following day.

Exercise 1. Anthrax (a).—

For this exercise a mouse is infected with anthrax bacilli. The infection is done in the following way: At the root of the tail the hairs are clipped off and the skin is held up by forceps. With the scissors a tiny incision is made just large enough to insert a platinum loop containing a loop-ful of anthrax bacilli. The mouse is kept in a special jar. Label the jar with the date of inoculation and the manner of infection.

Second Day**1. Further preparation of solutions**

From the alcoholic solutions the following preparations are to be made:

1. *Carbol Fuchsin (Ziehl's solution).*—5 per cent carbolic acid (from liquefied carbolic acid, which contains 100 parts of carbolic acid and 10 parts of water); 100 c.c. Saturated alcoholic solution of fuchsin; 10 c.c.

2. *Dilute carbol fuchsin.* The preparation as indicated under No. 1, ten times diluted. Both solutions remain perfect for several months.

3. *Loeffler's alkaline methylene-blue:* 1 litre of a 0.01 per cent potassium hydroxide solution;¹ 300 c.c. of saturated alcoholic solution of methylene-blue. It is advisable to prepare a large quantity, as the solution becomes more effective after a certain time, and to dilute this solution for staining.

4. *Aniline water methyl violet:* Shake 4 c.c. of anilin oil with 100 c.c. of distilled water for a few minutes. Filter the mixture through a wet paper. The filtrate must be absolutely free from aniline oil and perfectly clear. To this solution 11 c.c. of concentrated alcoholic methyl violet is added. On the top of the bottle a funnel with a filter paper is placed, so that the solution can always be filtered before using. This solution deteriorates rapidly.

5. *Far better results are obtained with Kutscher's gentian violet:*

Saturated fresh aniline water	} equal parts
Absolute alcohol	
Carbolic acid	
Gentian violet to saturate the solution	

This solution remains perfect for several months.

This solution should be diluted for use in such a way that a fine shiny membrane appears on the diluted stain (usually 8 to 10 drops to 5 cm.).

¹ 10 c.c. of a 1 per cent solution of potassium hydroxide plus 990 cm. distilled water equals 0.01 per cent, solution KOH.

6. *Carbolic thionin-blue* (Nicolle). Dissolve thionin-blue 1 gram, with carbolic acid 2.5 grams in distilled water 100 grams. Before using, dilute with equal quantity of distilled water and filter.

NOTE: The stock solutions are kept on the laboratory tables in small drop bottles. Never forget to label the bottles with the name of the solution and the date of preparation. With certain exceptions, the best results are always obtained when staining with weak solutions for a long period rather than with a strong solution for short time.

Exercise 2.

Simple Methods of Recognizing and Isolating Bacteria (a)

Material used: Infusion of manure, hay, and potato.

1. *Examination in hanging drop*

Surround the glass ring on the hollow ground slide, with a small amount of vaseline. Flame the platinum wire needle and part of the holder to destroy the germs which may be hanging to it. The platinum wire heats most rapidly when held nearly vertically in the flame. It cools in a minute when it is held in the air. The loop is plunged into the liquid manure and a minute droplet is placed on the middle of the cover-slip (which has been previously cleaned and held by forceps). The platinum loop is flamed again. The droplet should have a diameter of only 2 to 3 mm. and be very thin. The cover-slip is now inverted with the drop downwards in the hollow of the prepared slide (hanging drop) and is slightly pressed to the slide, so that the space of the ring is closed air-tight by the vaseline.

Examine the preparation first with the high-power dry lens, close the diaphragm of the Abbe's illuminating apparatus and use the concave mirror. As soon as the edges of the drop are observed distinctly, put a small amount of cedar oil on the surface of the cover-slip and move the immersion lens gradually towards the preparation. Focus the preparation, when looking through the ocular, by means of the fine adjusting screw. After a short period bacteria will be observed if the drop is in the center of the lens. When the drop is out of focus droplets only are seen, which have settled down on the cold walls of the chamber, which is saturated with moisture. It is often advisable to focus these droplets and then move gradually into the centre of the preparation, to find the bacteria. It happens frequently that when experimenting in the manner described, the cover-slip may break, which may be avoided by gently moving the slide to feel the pressure. If this accident should occur the entire slide is put in lysol solution and the immersion lens is cleaned from the cedar oil by means of lens paper and then disinfected with a soft linen cloth soaked in lysol solution.

In the hanging drop are observed:

(a) Bacillary-formed, rod-shaped micro-organisms of different lengths, often single, often in chains, often in filaments. They are non-motile or motile. If the movements are in one direction only, then they are motile. If the bacteria dance in one spot we call it Brownian movement.

(b) Spherical bacteria, cocci of different sizes, non-motile. They are usually single, often double (diplococci), cubic (sarcina), or quadruple (tetrads), often in chains (streptococci), or in irregular clumps, (staphylococci).

(c) Spiral or curved bacteria are either very small or extremely large, very motile, and can be observed only at the edges of the preparation, where they usually remain steady for a few seconds.

(d) Protozoa (seldom).

(e) Debris.

Open the diaphragm and the entire picture will disappear. Keep the preparation until the end of the exercise and compare with the stained preparation. Always keep in mind that unstained preparations alone satisfactorily demonstrate the motility of bacteria. The preparation is destroyed in the following way: By means of forceps, the cover-slip is carefully taken away from the slide and is put in the lysol solution. Avoid the possibility of the droplet touching the edges of the vaseline ring. The slide can then be cleaned with a towel.

Probable mistakes.—(a) When moving up and down, the cover-slip may become detached from the slide; the cause being that the cedar oil is too thick or the vaseline is too thin.

(b) When focusing, the bacteria appear indistinct. Cause: The drop is too thick.

(c) The bacteria are indistinct. The field is dark. Cause: The objective is not in focus, or a wrong lens is used.

(d) The bacteria appear indistinct, surrounded by a light area. Cause: The drop is dry, because it has not been sufficiently protected from the air by the vaseline.

(e) Large, light crystal-like figures appear. Cause: The vaseline has been focused.

2. *Examination of stained preparations*

A minute droplet of manure extract is taken with the platinum wire loop and placed on the cover-slip which is held by a cover-slip forceps. By means of the curved needle it is spread on the surface of the cover-slip. This process has to be done very carefully, otherwise the structure of the material may become destroyed. Flame the loop. Let the preparation dry, which may be easily accelerated by passing the film high

above the flame. When it is dry fix the bacteria to the cover-glass by passing it, film upward, three times through the middle of the upper half of the gas flame. By this means the bacteria become fixed (adherent by the coagulation of albuminoids) and at the same time, if we are not dealing with spores, the bacteria are killed. In cases where urine or other fluids, which contain no albuminoids, are examined, it is advisable to mix the material with a small amount of egg albumen and boric acid. After fixing and cooling, the preparation is ready for staining.

Preparations as described should be stained with (1-10) diluted carbol fuchsin, undiluted carbol fuchsin, Loeffler's methylene-blue or undiluted carbol thionin.

With the drop bottle, a few drops of staining solution are placed on the film of the fixed preparation, which is held horizontally with the forceps or left resting on a tray. Allow the stain to act for twenty seconds to three minutes. Rinse off in water. Place the preparation, film downward, on a clean piece of filter paper, and dry it. Mount in cedar oil or balsam. The latter medium causes the preparations to decolorize rather rapidly. Examine with immersion lens, flat mirror, open diaphragm and best light.

1. Observe in the slide stained with diluted carbol fuchsin, bacilli, cocci, spirilli and debris. The bacteria are intensely red; besides these, badly stained organisms are also visible; they have been dead for a long time and the chromatin has been soaked out.

2. In the preparation stained with methylene-blue, the bacteria and the debris are far more distinctly visible than in the first preparation. This is characteristic of methylene-blue stain and it can, therefore, be recommended for all specimens where coagulable material, tissue, serum, etc., surround the bacteria.

3. In the preparation stained with undiluted carbol fuchsin, the debris is stained very heavily and obscures practically all the bacteria. The specimen is, therefore, overstained and this method for such preparations is useless.

4. In the preparations stained with carbol thionin, the bacteria are observed to be stained dark blue, often with a violet tinge.

The preparations should be kept in a slide box and can always be used (at least until the end of the course) for comparison. Preparations which have become decolorized are put in xylol and then in absolute alcohol and can be restained with any stain.

Isolation and Growing of Bacteria

In the preparations considered different kinds of bacteria were visible. If we intend to study one special kind it is necessary to sep-

arate it from all others and at the same time allow micro-organisms to multiply free from adherent contaminations. The first step for isolation of a pure culture is as follows:

“Plating” of Bacteria.—

Take three gelatin tubes and liquify the contents in hot water at a temperature of 45 degrees C. Let the gelatin cool to 37 degrees C, or lower, until the tubes can be held comfortably in the hand. Do not be hurried, as gelatin coagulates only at a temperature of about 23 to 27 degrees C. This cooling down is necessary, as otherwise the bacteria which are brought into the liquified media are killed by the high temperature.

When the right temperature is reached, heat the platinum loop in a gas flame, take out the cotton plug from one test-tube and hold it between the fingers in such a manner that the plug or the tube end projecting *outward does not touch anything* during the inoculation process. Infection from the air is less likely. Carefully flame the mouth of the tube, then bring a loopful of manure mixture in to the gelatin, which is kept in the tube in a nearly horizontal position. The droplet from the needle is placed inside the test-tube at a place where it just reaches the fluid of the tube, and the material gently rinsed from the loop. Place the platinum needle, after flaming, back in its stand. Mix the droplet by carefully shaking the gelatin. In doing so the bacteria are uniformly distributed. If now a plate is poured, the fluid coagulates, and the bacteria become fixed *in situ*, and each develops singly, as a colony. In our particular case, the bacteria are so numerous and the colonies so close together, that an isolation would be impossible. We must prepare, therefore, dilutions from the first tube (original) into a second (I, dilution) and into a third tube (II, dilution). Flame the loop and transfer three loopfuls of gelatin from the original tube into the second and mix by carefully shaking. Again flame the loop and transfer three loopfuls from second tube to third tube and mix as described before. After all the tubes have been inoculated, pour the gelatin into Petri dishes first carefully flaming the mouth of the tube, and, after quickly cooling, raise with the left hand the edge of the cover on one side of the Petri dish sufficiently to allow inserting the mouth of the tube. After the gelatin is poured out, replace the cover immediately.

When “plating,” the dishes should stand horizontally and the bottoms of the dishes should be uniformly covered by gelatin, which can easily be brought about by moving the contents slightly over the uncovered places. Cool and coagulate the gelatin on a horizontal plate which is

kept cold by iced water, Label and number the Petri dishes to correspond with the dilutions in the tubes, in the following way:

Manure, July 19, 1913.

Alkaline gelatin.

Original, I; 1:1 (1st dilution); 1:100 (2nd dilution).

In the same way, two series of agar plates, of three tubes each, are made. The agar melts only at a temperature of 98 degrees C and coagulates at 37 degrees C. Take care, therefore, when cooling down the agar, that the moment for inoculating is selected when the temperature is just above the coagulating point. The bacteria can be brought easily into the media at this moment without the danger of killing them. This work can be simplified by leaving the tubes in a water bath until the thermometer reaches approximately 40 degrees and then the test tubes are inoculated and the plates are poured. If there is no thermometer available, cool the melted agar tubes until they can be held comfortably in the hand.

Possible errors.—An irregular surface of the agar shows (a) the agar was not entirely liquified (not heated long enough) or (b) when cooling down the tubes became partially coagulated. This process of making agar plates is commonly used at the present time, instead of gelatin plates, in countries where the room temperature rises over the melting point of the gelatin and no cold incubator is available.

The gelatin plates and one series of the agar plates are kept in the drawer or in the cabinet of the working table. The other series are incubated at 37 degrees. The agar plates are generally put in the incubator after cooling and inverting the plate to avoid the smearing of the colonies through falling drops of condensation water.

Exercise 1. *Anthrax* (b).—

The mouse does not show any symptoms of disease; only a slight edema is visible at the seat of inoculation. If the mouse is dead, then a reinoculation of a fresh one should be made.

Hay Bacillus. Place in a beaker a small amount of hay, cover with water; boil once for two minutes and then keep at 22° C.

Third Day

Exercise 2. *Simple methods of recognizing and isolating bacteria* (b).—

1. *Microscopic examination of the plates*

First about six to ten agar tubes are liquified and solidified in a slanted or inclined position. This is done in the following way: Put a few tubes of agar in a test tube stand, in a water bath and boil until

the agar is liquified. Lay the tubes on a tray, so that the surface of the agar will be about 4 cm. long and allow it to cool. When the agar is coagulated it is ready for use. This process of making agar slants always before use is more advisable than to keep them in stock, particularly as certain organisms do not grow well on dry media, and slanted agar dries out much more quickly than agar, which is not slanted.

On the agar plates kept at 37° C, several colonies have developed. If there is no growth, the platinum loop was still hot when brought in the manure, and the bacteria have, therefore, been killed. We see colonies both on the surface of the agar, and in the depths of the media. The first ones are usually large, round, the surface flat or rough, grayish, gray-yellowish or yellow with irregular spots in the middle. If the superficial colonies are abnormally large and very thin, the condensation water has spoiled the colonies. The colonies in the depths are round, usually filamentous, amoeboid, wedge-shaped and so on. On the second and third plate there are usually fewer colonies than on the first plate. One plate, where the colonies are nicely separated and isolated from each other, is selected for further examination. On the agar plates and the gelatin plates left at room temperature or 23 degrees C perhaps only a few small colonies are visible. The temperature was not suitable for their growth.

Examination at sixty times enlargement.—The cover of one plate is taken off and the bottom plate is placed openly under the microscope and examined with half-shut diaphragm and 1-3 objective. The appearances of the colonies are varying. They are found colorless up to a dark brown; finely granulated, or rough with irregular deposits. The edges, or the borders, are undulated, lobulated or "entire." In the text-books on special terminology, a full description with key is given and it is advisable for the student in this exercise to give a full description of two or three of the colonies. On the gelatin plates many small colonies are seen on the first plate (do not mistake scratches or bubbles in the glass or medium, respectively, for colonies).

Examination with high power.—Examine the bacteria of different colonies:

(a) In hanging-drop preparations. The preparation is made in the same way as on the second day. Take a cover-glass as before and place a loopful of bouillon or sterilized water on the centre. The platinum needle is flamed and a *very small amount of the bacterial growth* is mixed in the bouillon, just sufficient to make the bouillon slightly turbid. The colonies tested are always marked with indelible pencil, ink, or glass pencil on the back of the plate. One observes in each drop only one type of bacteria, which may be cocci bacilli or spirilla. The cocci:

One observes the size and their structure; with bacilli, the movements are particularly noted; they may be non-motile or move slowly more or less in one direction or irregularly. Often the bacteria hang together, again, they may form filaments.

(b) Stain several colonies (which means the bacteria of these particular colonies) with diluted carbol fuchsin. This is done in the following way: Place a small water droplet with the platinum loop on a cover-slip (the right size of the water droplet is obtained when putting the top of the loop under a strong water current), then flame the platinum wire *needle*, take a small amount from the surface growth, distribute it equally in the water droplet. Stain as usual. Examine with the immersion lens and observe the size and the structure of the bacilli; particularly note if they have a capsule, or if they stain regularly, deeply or faintly, or irregularly; is the centre lighter than the periphery or end? The background of the films should be entirely clear, otherwise we know that a certain amount of agar has been smeared with the bacterial colony.

(c) Stain similar preparations with carbol thionin, and methylene-blue.

2. Subculture from Colonies

Three or four of the marked colonies are transplanted on agar slants in the following way: Remove the cover of the Petri dish, invert the bottom part so that the inside is covered. Take the agar tube, to be used between the third and little finger; hold the plate vertically with the thumb and second finger to avoid air contamination; flame the mouth of the test-tube as described and remove with the flamed needle a small amount of the colony and make a horizontal superficial stroke on the agar. Always hold the agar tube inclined. Another method is to leave the plate on the table and raise with the left hand, the edge of one side of the Petri dish, and, while holding it, charge the needle from the colony. In a similar way agar and gelatin *stab* or *stick* cultures are made. The latter are prepared by touching the colony very superficially with the flamed and cooled platinum needle; push the impregnated needle down through the centre of the upright agar or gelatin. The stick should not go to the bottom of the tube. When taking the needle out, carefully observe if a small superficial hole remains at the top of the stick, as this might lead to a misinterpretation of liquefaction. No tubes in which the gelatin shows cracks after inoculation should be used, because they are misleading. Label all the tubes with the number of the colony, the date and the media used. Incubate the agar cultures at 37 degrees C and keep the gelatin cultures at 22 degrees C.

The results concerning the various micro-organisms are noted in a table of the following pattern:

No.	Form	Motility	Gram. stain	Spore formation	Gelatin plates		
					Shape	Color	Lique- faction
	Small coccus, irregular position	—	+	—	irregular, 2 mm.	Yellow	
		Agar plate	Gelatin stab		Agar stab		
		Granulated, 2 mm.	Superficial liquefaction needle track; yellowish clumps		Yellowish surface growth, needle track, yellowish clumps		

Exercise 1. *Anthrax* (c).—

If the anthrax mouse is not already dead, kill it with chloroform.

By means of forceps, the mouse is taken out of the glass jar and is pinned by all four legs with strong pins, on a piece of wood. The instruments for the postmortem are sterilized. The more utensils there are at disposal the better, because the instruments should be changed very frequently and the opening of the different cavities should be done with fresh scissors and forceps. The instruments should never be laid down on the table, as they would infect the table and become contaminated with other germs. The mouse is washed with 60 per cent alcohol to avoid flying hairs. Then make an incision from the larynx to the pelvis. The skin is removed in a way that will be demonstrated. With a fresh knife an incision is made into the muscles of the abdomen, from the xiphoid cartilage down to the pelvis. The ribs are cut through on both sides. The diaphragm is removed and the thorax exposed. With the exception of an enlargement of the spleen and a general hyperemia, nothing in particular is found. (White mice may have a large spleen under normal conditions.) Foci in the liver are probably due to coccidia or taenia. To collect the heart-blood under sterile conditions, flame an old knife until red hot, touch the pericardium with it, perforate the ventricle at seared spot with a sterile glass pipette. The aspirated blood is used for the examination.

By means of the forceps, after the trachea has been divided, the organs of the thoracic cavity are loosened, removed, and put in a sterile Petri dish. The liver, spleen, and kidneys are also removed and placed in a Petri dish. The instruments are boiled, the needles flamed and the carcass destroyed (put in a vessel with crude sulphuric acid). The piece of wood is flamed or boiled.

One-half of the lung, the liver, spleen, and kidneys are kept for sections.

1. *Examination of the blood and organs*

Hanging drop.—Mix a small amount of heart-blood in a droplet of saline with the platinum needle. Observe, especially along the border of the droplet, red blood corpuscles and long filaments of bacteria which lie in different directions. They are non-motile. When examined with a dry high-power lens the bacilli are just visible.

Stained preparations.—A drop of blood is placed on a cover-slip and with the slightly curved needle is smeared equally over the surface. The organs are cut with scissors and the cut surface is smeared over the cover-slip. When dry, they are fixed in the flame and stained with dilute carbol fuchsin. Large bacilli in short chains are seen, with sharply rectangular edges. Occasionally the chains are surrounded by unstained areas. No filaments are visible; only when the preparations are over-stained we are able to see capsules. Occasionally the capsule is surrounded by a light area, which is due to contraction of the albuminous material surrounding the bacteria. Some rods do not stain; they are dead. No spores are visible because they are never found in the animal body.

The nuclei of the white blood corpuscles are very distinctly stained. Occasionally the chromation of the nuclei is smeared out in filaments, which may be mistaken for bacteria. Small, dark globules are staining deposits. Stain several preparations with methylene-blue and carbol thionin. With the latter solution stain only a few seconds and examine them mounted in water, instead of using Canada balsam. The capsule of the bacilli is then usually very distinct.

2. *Special Staining for Anthrax Bacilli*

1. *Gram's method of staining bacteria.*—

Prepare the slide or cover slip, as already described. Stain it with methyl violet or Kutscher's gentian violet (dilute stock solution) for three minutes. Rinse in water, cover with Gram's iodine solution twice for one minute, then decolorize with absolute alcohol or 95 per cent until no clouds of color are washed away. The decolorization can also be done in a watch glass. Dry the slide, mount in cedar oil and examine.

Three other preparations are stained in the same way, but are restained, after decolorization with diluted carbol fuchsin or eosin, or a 3 per cent safranin solution. Rinse in water; mount in cedar oil; examine. All the bacilli are stained deep violet and the ends are more rounded. In the restained preparations the tissue cells have taken up the contrast stain and appear red or yellow-brownish.

Cause of error.—The bacilli may be irregularly stained and show only black granules, which means that the staining solution is decomposed or decolorization was too long.

2. Capsule stain.—

(a) The staining is done, according to John's method, with a 2 per cent aqueous solution of methyl violet, by heating until steam evaporates. Afterwards rinse the slide with water and bring the films for six to ten seconds in contact with a 1 per cent aqueous solution of acetic acid, then clean carefully with pure water. Examine first in water; dry the upper surface and add cedar oil for the examination. The bacteria are blue and have distinct capsules.

(b) *Safranin* (Olt's method). Stain the preparation with a 3 per cent solution of safranin, at room temperature, for three minutes. Rinse in water and examine as described above. The bacteria are red, the capsules yellowish. The method is not always successful.

(c) "Giemsa" solution. With this solution the bacteria can be demonstrated in a distinct manner. Fix the smear five to ten minutes in absolute alcohol and ether, keep in a watch glass or slide stand. Stain on a glass tray with dilute "Giemsa" solution (one drop of stain to 1 c.c. of neutral distilled water (1 to 5 drops of sodium carbonate solution 1-1000) for twenty to thirty minutes. Rinse in water; dry; mount in cedar oil. When on slide, examine without cover-slip. Use also the capsule stain of Muir. (p. 111.)

NOTE: In artificial cultures capsules can never be demonstrated; therefore if the time is limited make some smears on cover-slips, fix them by passing through the flame and keep till the next day. The exercise with these different stains can easily be done another day, when more time is available.

3. Pure Cultures of *Anthrax Bacillus*

If stab or streak cultures are made from the organs, one may obtain contaminated cultures, due to foreign bacteria that are easily overlooked by the microscopic examination; therefore, it is better to prepare agar and gelatin plates as usual. For the inoculation of the agar, a loopful of heart-blood, and for the gelatin a small piece (the same size as the

platinum loop) of the different organs (spleen or liver) are carefully distributed by rubbing the material along the walls of the test-tube just above the fluid. At the same time a culture in a hanging drop is made. For this purpose the hollow ground slide and two cover-slips are flamed and put in a sterile Petri dish. On one of the cover-slips a droplet of bouillon is infected with a trace of heart-blood. Close the hanging drop tightly with vaseline, as usual. Put the preparation in a Petri dish and incubate with the agar plates at 37 degrees, the gelatin plates at 23 degrees.

4. Preparation of Histological Sections from Anthrax Mouse

The organs reserved for this purpose are put in a 10 per cent formalin solution and kept in a dark bottle (otherwise the formalin disintegrates). They are later imbedded, cut, and stained.

5. Hay Bacillus

In the beaker with the boiled hay infusion, a fine pellicle has formed. In the hanging drop and in stained preparations, bacilli of the same size as the anthrax bacillus are seen. The ends are rounded. Numerous spores are present. Pour gelatin and agar plates.

Liquefy a tube of agar; pour a plate and place the same in the incubator (see Exercise 1 (d), 5).

Fourth Day

Exercise 2. Simple methods of recognizing and isolating bacteria (c).—

1. On the agar plates kept at 37 degrees the colonies are enlarged and new ones have appeared. It may happen also that one colony has covered the entire plate.

2. On the agar plates kept at 23 degrees the growth is somewhat better today, but never equals that of the incubated ones. If colonies appear, observe if they are pigmented, or if the pigment runs over into the surrounding medium.

3. On the gelatin plates a remarkable growth is present. Several colonies have liquefied the gelatin in a more or less distinct manner. Note that, from such a colony, subcultures can be made only when one is thoroughly convinced that no other colonies are lying in the liquefied zone. The appearance of the colonies is quite different from those on the agar. Examine, at sixty times enlargement, hanging drop, and stain. (This is not liquefaction. Agar never liquefies.) *Never incline the test-tube so that the condensation water runs over the colonies.*

4. On the streak cultures the growth of the bacteria is visible at times only along the streak, again over the entire surface, often only in small colonies. Note if color and appearance are identical with those of the plates. At the bottom the condensation water may be slightly turbid. (This is not liquefaction. Agar never liquefies.) *Never incline the test-tube to that the condensation water runs over the colonies.*

5. When examining the stab cultures, one has to consider: (a) The growth on the surface; (b) the growth in the depths of the media. On the agar stab cultures the surface resembles more or less the growth on the surface of the plates. The stab may be filiform, tuberculate or villous. The further the needle track runs down into the depths, the less the growth of the bacteria is visible, because less material and less oxygen is present. Occasionally gas bubbles are seen. In the gelatin stab cultures the surface growth is similar to that on the agar plates, or the gelatin is liquefied and shows napiform or sacculated holes. If the whole gelatin is spotted with colonies, then proof is given that the gelatin was inoculated before it was actually coagulated.

Exercise 1. Anthrax (d).

1. On the agar plates several uniform colonies are visible. The superficial ones are rough, furlike, with irregular margins. In the depths the branching is more distinct than the actual colonies. Occasionally superficial, round colonies are due to germs from the atmosphere. At sixty times enlargement, single filaments which give the colony a waved, rounded appearance, are visible. Also the wavy structure is seen in the inside of the colonies. The filaments lie parallel to each other. Along the borders several branches creep centripetally. Occasionally the filaments appear non-homogeneous and show dark spots (spores).

Stain by Gram. Stain also for capsules. No capsule can be demonstrated, in colonies, on artificial media. Capsules are characteristic of anthrax in the animal body.

2. *Making impression Preparations* (Klatsch prepare).—A cover-slip carefully cleaned and flamed (by passing it once through the Bunsen flame) is put on top of one of the colonies on the agar plate, so that the superficial colony lies in the centre of the slip. Touch it very gently with the forceps. One minute afterwards it is removed with a pair of forceps, being supported by a needle on the opposite side, to avoid dislocation from its original position. The colony sticks to the cover-slip. Let the cover-slip dry for one-half hour to an hour. Fix it in the flame and stain it very gently and carefully, because the bacteria are easily washed away. Examine with low and high power. The bacilli are kept in their position as they were on the colony. Perfect preparations of anthrax bacilli are obtained only when the agar is dry.

3. *Examination of the culture in the hanging drop.*—With low power, the appearance is similar to that of a colony in the depth of agar. With high power, one observes, between degenerated blood corpuscles, filaments with distinct sporulation. Occasionally, also, short bacilli and cocci are visible, a proof that the culture was contaminated.

4. *Gelatin plates.*—With the naked eye, nothing is visible. Examine the plate. In focusing on one of the small organ pieces, filaments and chains are usually seen; perhaps also small colonies may be detected.

5. *Method of staining spores.*—If spores are found in the hanging drop, a "spore stain" can be tried. If not, inoculate an agar plate prepared the day before (and which has become dry) with the material of a colony, in the way that will be demonstrated. Also inoculate one tube of peptone-free agar with material of the same colony. Stain twenty-four hours afterwards, and not later, otherwise the spores will have dropped out of the chains and become invisible.

As was shown before, spores cannot be stained by usual methods. The highly resistant membrane must be macerated, which is usually done with a 5 per cent solution of chromic acid. Make five cover-glass preparations, dry and flame, as already described, and cover the film surface with chromic acid. Let the chromic acid act on one cover-slip one minute; on the second, three minutes; on the third, five minutes; on the fourth, ten minutes; and on the fifth, fifteen minutes; because the action of the chromic acid must be determined in each particular case. Rinse in water, cover the surface of the film with undiluted aniline water fuchsin (prepared in the same manner as aniline gentian violet) and hold the preparation over the flame until steam is given off, for about one minute. Repeat three times. Decolorize by immersing it in a watch-glass containing a 1 per cent solution of hydrochloric acid in alcohol. Rinse the preparation in 60 per cent alcohol for about half a minute. Repeat if the bleaching process of the film is not decolorized thoroughly enough. Carefully wash the preparation, counterstain it with Loeffler's methylene-blue for about two minutes. This has to be done because the bacteria are usually affected by the boiling process.

Make subcultures from the superficial plate colonies on agar streak, and agar and gelatin stab cultures. Inoculate bouillon and potatoes, the latter by making a superficial streak.

The organs which have been fixed in formalin are washed in running water for twenty-four hours, by placing them in a bottle which is closed in such a manner that the tissues cannot be washed out.

6. *Hay Bacillus.*—On the gelatin plates are colonies similar to the anthrax bacillus. On the agar plates the margin of the colonies is very irregular. Make sub-cultures.

C. EXPERIMENTAL PATHOLOGY

All experiments will be conducted by a group of six students under the immediate supervision of the instructor. The preparatory work will be done as far as possible on Tuesday mornings, the cases followed as directed, and the results demonstrated to the whole class when most convenient. In all experiments there must be surgical cleanliness of instruments as well as of the hands of the operators, and thorough preparation of the animal in respect to asepsis and anesthesia.

Experiment 1: Neuropathic atrophy.—The object of this experiment is to demonstrate an atrophy of muscle following section of a motor nerve, the reaction of degeneration, and histological alterations in the muscle, myelinic degeneration in the nerve, and axonal alteration in the nerve cell. A young rabbit is etherized. The posterior surface of one hind leg is shaven, washed with soap and water, followed by alcohol and ether, and along the line of incision a few drops of tincture of iodine is spread. Arrange sterile towels about site of operation. Make incision, expose nerve as high as possible. Grasp it with forceps and pull it out from above. Cut out a segment of uninjured nerve for staining, as a control. Close the wound by suture, apply celloidin dressing. Examine for reaction of degeneration in muscle, at intervals. After three weeks chloroform the animal, expose and examine the muscle of both legs; note difference in size, color and texture of muscles. Preserve sections from normal and abnormal muscles in Zenker fluid, alcohol, and formalin. Place bits of the degenerated nerve in Muller's fluid and prepare by the Marchi-Allgeri method for demonstration of oleic fat. Also put a segment in Zenker's fluid. Dissect out carefully the lumbar cord and preserve in 95 per cent alcohol for Nissl's stain. ff

Experiment 2.—The object of this experiment is to demonstrate cloudy swelling. Inject 0.001 gram cantharidin dissolved in acetic ether (ethyl acetate) subcutaneously daily. Examine the urine daily for albumen and sugar, and for blood and tube casts. After death make frozen sections of kidneys and liver and examine sections in normal salt solution and in dilute acetic acid.

Experiment 3.—Fatty degeneration is produced by injecting subcutaneously 0.020 gram yellow phosphorus dissolved in oil of sweet almond. Examine organs after death, especially liver. Make frozen sections after fixation in formalin and stain with Scharlach R by Herxheimer method. Also preserve tissue in Zenker's fluid.

Experiment 4: Obstructive jaundice.—Ligate the ductus choledochus in a dog or a cat. Observe jaundice of skin and sclera. Examine urine for albumen, casts, and bile pigments.

Experiment 5: Hematogenous jaundice.—Bleed a rabbit from the carotid artery with a sterile tube, transfer about 10 c.c. of blood into an equal quantity of sterile distilled water. After complete laking, centrifugalize and to the decanted solution add sufficient salt to make the solution physiologically normal. Re-inject this solution into the ear-vein of a rabbit. Note the occurrence of hemoglobinuria, and later the appearance of bile pigments in the urine. Rattlesnake venom may also be used to destroy red blood corpuscles, giving 0.001 gram intravenously three days before demonstration.

Experiment 6: Calcification.—Ligate one renal artery of a rabbit. Autopsy after four weeks.

Experiment 7.—Inject subcutaneously daily 10 c.c. 1-1000 solution of mercuric chloride for three days. Kill after four days. Examine organs, particularly kidneys.

Experiment 8.—Inject a thin suspension of fine cinnabar intraperitoneally and subcutaneously in different regions. After twenty-four hours, examine and trace the distribution of the granules. Make microscopic section to demonstrate phagocytic action.

Experiment 9: Necrosis from loss of blood-supply.—Ligate a large branch of superior mesenteric artery in a dog. Study the peristaltic alterations. Close abdomen and after two days, sacrifice the animal, keeping careful clinical notes in the interval.

Experiment 10.—Necrosis due to chloroform is produced by keeping a guinea-pig under the influence of chloroform (most conveniently in a box through which the dilute chloroform is forced by compressed air) for several hours on successive days. Kill the animal and make sections of organs, especially the liver. Preserve tissue in formaldehyde solution, also in Zenker's fluid.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Atrophy is a decrease of the parenchyma and usually of the size of a part due to physiological causes or to disease. Atrophy is differentiated from hypoplasia, which is an underdevelopment and which may be hereditary or acquired. Abiopathy is a condition of subvitality of a part, which develops normally for a time but then undergoes regressive changes. The condition is hereditary and the central nervous system, as in Huntington's chorea, may be used as an example. The occurrence of arteriosclerosis in very young individuals may also be so interpreted. Depending on the cause atrophies are sub-divided into:

Physiological: normal recessive changes, as the atrophy of the thymus or of the sex-glands at appropriate age; senile atrophy: which is in part physiological but may come on prematurely and affect organs irregularly; atrophy of disuse; atrophy of malnutrition; pressure atrophy; neuropathic atrophy; toxic atrophy: infectious, malignant new growths, X-rays, etc.

Discussion of the gross appearance and microscopic alterations in atrophic tissues.

Brown Atrophy.

Demonstration.—It is essential before deciding what is abnormal to know the normal. With this in view we will make an autopsy on a normal rabbit. Post-mortem changes may be mistaken for pathological alterations, so an animal which has been killed forty-eight hours previously will also be demonstrated. Sections of tissue are to be taken from these animals and, after fixation in Zenker's fluid, sectioned and stained. These will then serve as a control for future work.

Second Day

Under the term cellular degeneration are included all morbid changes which may result in cell death. They may be the result of chemical or bacterial irritants, trauma, extremes of temperature, etc. The degenerative changes may show in the cytoplasm or nucleus or both. The principal forms of degeneration are:

(a) Parenchymatous degeneration, or cloudy swelling; closely related to this, hydropic degeneration. Reaction of degeneration.

(b) Fatty degeneration (not to be confounded with the above). Lipoidal degeneration, myelinic degeneration.

(c) Pathological keratinization.

(d) Mucoid degeneration.

(e) Colloid.

(f) Hyaline degeneration—Zenker degeneration.

(g) Amyloid degeneration.

(h) Glycogenous infiltration.

Third Day

Necrosis: Death of the body as a whole. Summary of post-mortem alterations, loss of heat, rigor mortis, post-mortem lividity, drying of body, clotting of blood, character of clots, putrefactive changes, microscopic changes, cytoplasmic alterations, karyolysis, karyorrhexis, pyknosis.

Focal necrosis; Colliquative necrosis; Fat necrosis; Adipocere; Gangrene: dry, moist.

Fourth Day

Jaundice is a staining of the tissues with bile pigment. Jaundice may be obstructive, i.e., due to reabsorption of bile from the dilated bile capillaries of the liver, where obstruction blocks the free passage of bile; or the jaundice may be hematogenous, due to the setting free of hemoglobin which is readily split into hematin and globin; the former is subdivided into hemosiderin, an iron-containing portion, and hematoidin, which is isomeric with bilirubin. This process occurs without the necessary intervention of the liver. Mechanical features which cause obstructive jaundice. Jaundice from acute liver degeneration. Cause of hematogenous jaundice. Influence of bile pigment on the kidneys and on the coagulation of blood. Icterus neonatorum.

Pigments: normal pigments, their occurrence and distribution. Those delivered directly from cellular activity—Melanin, its distribution normally. Freckles. Skin irritation. Vagabonds disease. Chloasma, Addison's disease. Relation of pigmentation to intra-abdominal tumors. Pigmented moles.

Hematogenous pigments: hemosiderin and hematoidin, in old hemorrhages—Pernicious anemia; Lipochromes: pigments of atrophy and of nerve cells; Hemochromatosis; Malarial pigmentation—Pseudomelanin; Hemoglobinuria: Paroxysmal—Infective.

SECOND WEEK

GENERAL OUTLINE

A. Bacteriology

First day.—Simple methods for recognizing and isolating bacteria (continued); Anthrax (continued); preparation of culture media (continued).

Second, Third and Fourth days.—Preparation of culture media (continued).

B. Infection and Immunity

Lectures on infection and conditions of natural immunity.

Experiment to demonstrate natural immunity and factors concerned in it.

C. Experimental Pathology

Experiments illustrating different types of inflammation and the repair of clean and infected wounds. (To be continued during next two weeks.)

D. Histopathology and Morbid Anatomy

First day.—Calculi and concretions.

Second day.—Technical methods: Fixation, imbedding, section cutting.

Third and Fourth days.—Acute inflammation.

First Day

A. BACTERIOLOGY

Exercise 2. *Simple methods for recognizing and isolating bacteria* (continued) (d).

The colonies on the plates have increased in number and are enlarged. Frequently the first gelatin plate is entirely liquefied. On the streak and stab cultures the growth has increased and, along the needle track, side branches, etc., have formed. The cultures are put back in the incubator.

Exercise 1. *Anthrax* (c).—

The colonies on the *agar* plates are larger (do not mistake dirt for colonies). With low power the appearance is the same as yesterday.

On the gelatin plates (avoid holding them slanted) several small gray colonies, which are liquefied, are visible. Under the microscope one will see that they consist of many filaments. On the *agar* streak cultures the appearance of the bacterial growth is similar to that on plates.

Gelatin stab cultures: The surface is liquefied. The surface growth is sunken in the hole which is formed by the liquefaction. Along the line of puncture, only, a partial growth is visible and shows irregular, villous branching.

On the *agar stab culture* we find a grayish felt-like deposit, with irregular margins. The stab shows small, irregular nodes and pearls along the needle track.

In the *bouillon cultures* the fluid shows a growth, like floating cotton. The bacilli are at the bottom; there is no pellicle formed. On the potatoes, along the streak of inoculation, a dull film has formed. Examine all these cultures in hanging drops, stain coverslips and make careful notes. Stain to determine if spores are present.

The *agar streak culture* is kept for later use. The cotton plug is cut and burned, then pushed in the test-tube, which is closed with melted paraffin. In this way the cultures may be kept for a long time. If subcultures are to be made, pour some sterilized bouillon on the surface and incubate for twenty-four hours.

Preparation of Culture Media

General directions.—

Weigh all boilers to be used and mark weight on tag attached to handle. When ever the required weight of contents is known, any loss by evaporation of water can be made up by putting boiler on scale, setting weight to amount of boiler plus contents, and adding water till the scale tips. This can be done at any stage of the preparation up to the filtering. After filtering, the filtered medium is weighed or measured again if further boiling is needed.

The basis of usual culture media is an infusion of fresh meat (1:1 or 1:2), or a solution of Liebig's meat extract (0.3–0.5 per cent).

Preparation of meat infusion.—

Take two kilograms of meat (beef or veal). Remove all fat and mince it carefully or, better, grind in a sausage machine. Mix the minced meat in a casserole with two liters of clear, boiled tap-water, and stir thoroughly with a glass rod. Macerate the entire mixture over the flame or in a water bath for two hours.

This process, just described, can be modified (if the time allows), by leaving the mixture of the meat (with the boiled tap-water) standing for twelve to eighteen hours in a cool place. For the routine work in the laboratory, the first process is quite satisfactory. Strain the liquid through a piece of cheese-cloth; squeeze out the juice still held in the minced and macerated meat. The resulting sanguineous fluid is boiled thoroughly for two hours.

Cool the filtrate and filter it through a wet filter-paper to retain the fat. The filtrate thus obtained should amount to two liters. If it does not, add enough water to make the desired quantity.

Beef broth (boullion).—Take 1000 c.c. of meat extract; add 1 per cent peptone (Witte) and 0.5 per cent sodium chloride (no sodium chloride is necessary when using Leibig's extract solution), and a sufficient quantity of normal NaOH to give the liquid a faintly alkaline reaction to litmus. The infusion is boiled in a water-bath for three-quarters of an hour and filtered hot. The filtrate should be perfectly clear. The color will vary according to the amount of blood pigment and to the length of time it is steamed or boiled. This boullion can be distributed in test-tubes or flasks.

The distribution of media into test-tubes is done by means of a funnel which is connected with a rubber tubing to a small glass rod and kept closed by means of a squeezing forceps. No test-tube should contain more than 10 c.c. Never tube more media than are actually needed for one week. The rest of the media can be kept in stock in tightly closed flasks. 100 c.c. bottles are more suitable for these purposes, because solid media can easily be melted. Then sterilize for thirty minutes in the Board of Health sterilizer, on three consecutive days or for thirty minutes in an autoclave at 120 degrees C. Label, for example, as follows: "Bouillon, 8-XI-1912."

It is frequently advisable to know the definite reaction of the culture media; for this purpose the titration method of Fuller is reliable:

1. Take 5 c.c. of boullion and place in a porcelain evaporating dish with 45 c.c. of distilled water, rinse the pipette in the water.

2. Boil for one minute.

3. Add 1 c.c. of phenolphthalein (0.5 solution in 50 per cent alcohol).

4. Titrate while still hot with N/20 NaOH in burette as follows:

- (a) Record the burette reading.

- (b) Allow the N/20 NaOH to run into the dish drop by drop until it is a bright pink color; boil again.

- (c) Record the final burette reading and subtract the first reading from it. The difference in cubic centimeters will represent the per cent of acidity of the medium. To neutralize 100 c.c. of the medium as much normal NaOH would have to be added as was required of the N/20 NaOH to neutralize 5 c.c. of the medium.

A standard of $+0.5$ to $+1$ to phenolphthalein, or slightly alkaline to litmus, has been adopted in America. In order to bring the entire amount of boullion to the desired reaction ($+1.0$), subtract 1.0 from the amount of N/20 NaOH solution used and multiply the difference by the number of cubic centimeters of boullion divided by 100. The pro-

ducts, if plus, represent the amount of N/1 NaOH to be added; if minus, the amount of N/1 HCL to be added. After mixing, test the reaction again in the same way and add alkali or acid, if needed.

Carbohydrate Broth.—Use either a stock solution of peptonized broth, prepared from Liebig's meat extract, or dextrose-free broth prepared in the following manner: To an infusion of meat (see above) add 5 to 10 c.c. of a pure twenty-four hour old culture of the *B. coli* or *B. saccharolyte* and incubate for fourteen to eighteen hours at 37 degrees C. Control the fermentation by carrying out indol tests (see later). Boil the broth to kill the organisms; filter. With such dextrose-free titrated broth, special carbohydrate broth is prepared by adding the carbohydrates required in the following proportions:

Glucose, Maltose, Lactose, Saccharose, Mannite, Glycogen, Dextrin, and Inulin 1 per cent; Sorbite, Dulcite, Arabinose, and Adonite, 0.5 per cent.

Dissolve the chemical carefully and put the broth either in Hill¹ or Durham tubes. Sterilize in the Board of Health sterilizer (*never in the autoclave*) for one-half hour for three consecutive days. Label carefully and keep in stock.

Potato Culture Media.—

Potatoes are thoroughly washed and rinsed in boiling water. Their eyes are removed, then discs of 1 cm. thickness are cut out and put in small sterilized glass dishes.

Another way to prepare potatoes resembles the form of ordinary solid media in test-tubes. Wash potatoes thoroughly, cut off ends and, with a borer, cut out cylinders from 3 to 4 cm. in length. Wash these potato-cylinders in cold water to prevent darkening; divide the cylinders into two equal parts by the diagonal cut; leave in cold, running water for twenty minutes. Place in six-inch test-tubes; in the bottom of each tube place some moistened cotton or a short piece of glass rod. Add about 1 cm. of water to each test-tube. The potatoes in the dishes and in the test-tubes are sterilized in an Arnold sterilizer for about fifteen minutes. The process has to be repeated the next day, increasing the time of sterilizing each day, say twenty-five to forty minutes. Wipe, label, and store the media.

¹ These tubes are a device extensively used for studying changes in reaction and collect part of the gas formed. The Hill tubes consist of a long test tube with the lower third bent at an angle, the whole resting on a base. The Durham tube is a long one-dram medicine vial, inverted inside a test tube containing a fluid medium.

Second Day*Preparation of culture media (continued)***No. 1. Gelatin.—**

Take flask 200 c.c. of beef bouillon infusion, add 30 grams of "Gold Label" gelatin (occasionally in winter time it is advisable to use 10 per cent). Put this casserole in a large water-bath (large casserole with water used as outside boiler and heat, with frequent stirring. Determine, and correct reaction. Cool to 50 degrees C and add, with frequent stirring, the white part of an egg dissolved in a small amount of water. Place liquid gelatin into the Arnold sterilizer and heat for forty-five minutes at a temperature of 100 degrees until the albumen is firmly coagulated and precipitates all the flocculent material which is suspended in the gelatin; determine and correct reaction. The medium is now ready to be filtered. This filtering process can be done through Chardin filter-paper or through cotton.

To Prepare a Cotton Filter.—Place a small bit of absorbent cotton in the bottom of a glass or enamelware funnel. Lay over this a piece of cotton larger than the palm of the hand, about one-half the thickness of the unrolled layer. Wet carefully with hot water, pressing the edges against the sides of the funnel. Wet with very hot water just before using.

The filter paper must be moistened with boiling water. It is advisable to keep the funnel warm by means of hot water, to avoid coagulation of the gelatin. The clear filtered and tested medium is filled into test-tubes in a manner that will be demonstrated (take care that the gelatin does not touch the sides of the upper part of the test-tube, otherwise the cotton plugs will stick to the tube and be the source of contamination). Sterilize the media for thirty minutes in the Arnold sterilizer, for three consecutive days, or in autoclave (not over 4 to 5 pounds pressure) for only fifteen minutes. Always have the gelatin solidified immediately, when sterilized, by placing the tubes in ice-water. By this means only, the coagulative properties of the gelatin remain unaltered. The reaction of this medium should be slightly alkaline or neutral to litmus, or 1.0 to phenolphthalein. Label the tubes carefully. Store in ice-chest to prevent evaporation as much as possible.

No. 2. Prepare 200 c.c. of gelatin which is distinctly alkaline or 0.5 to phenolphthalein.

No. 3. Preparation of Milk.—

Distribute fresh or "certified" skimmed, fat-free milk in test-tubes, in each about 10 c.c., and sterilize them in the same way as the potatoes. It is always advisable to have the cream entirely removed. This can

be done most satisfactorily by centrifugalizing the milk or by keeping the milk in the ice-chest and removing the lower layers. The reaction of the milk should be amphoteric or 1 per cent acid. An addition of 5 per cent litmus solution is recommended. Sterilize for five minutes at 120 degrees, or for 15 minutes on four successive days.

No. 4. *Litmus Whey*.—

Fifty c.c. of milk is heated and 1 c.c. of a 10 per cent hydrochloric acid solution is added. The sediment is carefully filtered and the filtrate neutralized (0.5) very carefully with sodium carbonate solution. Sterilize for one to two hours in the Arnold sterilizer, and filter again. Should the fluid not become clear, filter again. The fluid should be clear and only slightly yellowish; brownish discolorization proves it too alkaline and the whey is useless. To this preparation add 5 per cent of litmus solution (Kahlbaum, Berlin). The fluid should appear violet; 5 c.c. of the fluid is filled in test-tubes and sterilized in an Arnold sterilizer for thirty minutes on three consecutive days.

Third Day

Preparation of Culture Media (continued)

No. 1. *Agar*.—

Take 500 c.c. of beef bouillon; cut 10 grams of shredded agar into small pieces and soak it in the broth. Put this mixture in an agate dish or flask; place it in the autoclave, or boil it over an open gas flame, with constant stirring, until the agar is dissolved, forming a homogeneous thick mass. This process of dissolving agar often takes several hours, therefore it is advisable to carry out this exercise on a day when ample time is available. Let the melted agar cool down to a temperature of 50 degrees to 51 degrees C; add the white of an egg and thoroughly mix it in the liquid agar. At the same time, test the reaction of the media and correct it by adding NaOH or HCl, as explained above. Place the agate dish, with the liquid agar, in the autoclave; then keep it for about twenty minutes at 120 degrees C, until the egg-albumen is thoroughly coagulated. Determine the reaction, and then filter. It must be observed that agar filters only while hot, and very slowly, through ordinary filter paper. It is therefore, advisable to filter through cotton by placing the liquid agar in a large funnel lined with cotton and supported by a large flask. The combination is placed either in an Arnold sterilizer or in an autoclave. The temperature of the sterilizers will keep the agar sufficiently hot to enable a less interrupted, rather slow filtration. The filtered medium should be perfectly

clear and of known reaction. Distribute the agar in test-tubes or small flasks; sterilize it in the autoclave for two consecutive days, about twenty to thirty minutes. If the agar becomes turbid after sterilization, it must be refiltered—it was not sufficiently heated when the egg was coagulated. Label and keep in a cool place.

No. 2. *Glycerin Agar*.—

This agar is prepared in the same manner as just described, with the exception that 5 per cent glycerin is added. The amount needed for the course is about 100 c.c.

No. 3. *Carbohydrate agar*.—

Prepare 300 c.c. agar as described above, using dextrose-free broth, and add 1 per cent sterile glucose or lactose or mannite when filtered. Sterilize in the Arnold sterilizer for twenty minutes, for three consecutive days to avoid caramelization of the sugar.

Fourth Day

Exercise 2. *Simple methods for recognizing and isolating bacteria* (continued) (e).—

On the plates no more colonies have appeared. Perhaps in the depths irregular figures are visible, which prove at sixty times enlargement to be star-shaped crystals, produced by drying of the agar. Make careful drawings of these elements, so they will not be mistaken for colonies again.

The plates are destroyed by boiling them in an enamel pot for forty-five minutes.

Preparation of Culture Media (continued)

No. 1. *Egg media* (Dorset and Lubenau).—

These media are used with great success for the cultivation of tubercle bacilli.

(a) Four absolutely fresh eggs are carefully washed with 5 per cent carbolic acid solution; then the shell is broken and the entire contents blown into a sterile beaker and stirred with a glass rod. Afterwards the mixture is strained through cheese-cloth and tubed. The medium is sterilized and coagulated at the same time in the manner described for serum (inspissated at 70 degrees C for two to two and one-half hours). It is often advisable to add glycerin (4 to 5 per cent).

(b) *Glycerin egg* (Lubenau).—Ten eggs are blown into a flask and 200 c.c. of glycerin bouillon (5 per cent neutral) added. The further preparation is the same as with plain eggs.

No. 2. *Blood serum.*—

The blood is usually collected from horses or cattle. By means of a trocar, collect the blood in a sterile glass jar in a way which will be demonstrated. Keep the blood in a cool place and collect the serum (which has been pressed out) with a sterile pipette and distribute it in small test-tubes. This serum can be tested for its sterility in the incubator. All the tubes showing growths of bacteria should be eliminated. For laboratory use it is advisable to keep serum always in stock. This is done in a quite simple manner. Distribute the collected serum in small medicine bottles and add to each bottle about $2\frac{1}{2}$ to 5 per cent chloroform. The bottles should be tightly closed, with rubber stoppers. Two months later the serum is absolutely sterile and can be used. When using it, withdraw from the bottle the amount needed, place it in a sterile Ehrlenmeyer flask and incubate it, to evaporate the chloroform. For the work in the course, the serum is given out from bottles prepared as described.

(a) Tubes of liquid serum are inclined and coagulated at a temperature of 70 to 75 degrees C. This is usually in a blood-serum sterilizer or inspissator. The atmosphere in these sterilizers must be saturated with moisture.

(b) *Loeffler's serum.*—Put sterile beef serum, in the quantity of 3 to 4 c.c. in each of several test tubes and add to each 1 c.c. of sterile, slightly alkaline glucose bouillon. Mix carefully, incline and solidify them at a temperature of 100 degrees C. The exposure to heat is longer on account of the bouillon which has been added. The tubes are tested, before using by incubating them. All tubes which show growths of bacteria are discarded.

No. 3. *Blood agar.*—

To tubes of melted, plain or dextrose nutrient agar cooled to 50 degrees C, add from 1 to 3 c.c. of defibrinated blood (rabbit, horse or dog), observing aseptic precautions. The blood is collated either from the carotid artery in a sterile flask with glass beads or with a syringe one quarter full of sodium citrate 1 per cent and sodium chloride 0.85 per cent. Shake syringe; put 1 to 3 c.c. mixture in each tube of melted agar. Heat twenty minutes in water-bath or paraffin oven at 56 degrees C to inactivate the complements, thus reducing the bactericidal power of the blood.

No. 4. *Alkaline Blood Agar.*—

Obtain about 100 c.c. defibrinated blood by collecting it (when drawn from an animal) in a bottle (500 c.c.) with a glass stopper, previously filled with glass beads and sterilized. Shake the blood for at least

15 minutes. Mix 100 c.c. of the strained blood (cheese-cloth) with 100 c.c. N. potassium hydroxide solution (5 to 6 grams KOH in 100 c.c. water) and sterilize in steam. Liquefy 70 c.c. ordinary agar and mix it with 30 c.c. of alkaline blood. Sterilize again for half an hour. For use, pour in large Petri dishes and place in the incubator at 37 degrees C for a few hours to effect proper drying. This can also be effected by heating the medium at 60 degrees C for 1 hour.

No. 5. *Peptone Stock Solution.*—

Sterilize five flasks containing each 100 c.c. of distilled water. Add 10 grams of peptone, 10 grams of sodium chloride, 0.1 gram potassium nitrate and 0.2 gram of sodium carbonate. For use, dilute as follows: 3 c.c. solution, 27 c.c. distilled water.

B. INFECTION AND IMMUNITY

Experiment on Natural Immunity

Frogs at ordinary room temperature (15 to 20 degrees C) are insusceptible to infection with *B. anthracis*. By raising the body temperature to that of mammals, 37.5 degrees C, they become susceptible. It is also claimed by Dieudonne¹ that frogs, at normal room temperature, may be infected fatally by a culture grown for several generations at 12 degrees C.

Take eight frogs and divide them in four lots which are treated as follows:

(a) Two frogs kept in a glass jar at 37.5 degrees C are left as controls.

(b) Two frogs at 7.5 degrees C are given, each, 0.1 of an agar slant of *B. anthracis* accustomed to growth at 37.5 degrees C.

(c) Two frogs kept at room temperature are infected as those in "b."

(d) Two frogs, kept at room temperature, are infected with a culture of *B. anthracis* that has been grown at 12 degrees C, for one month or more, with frequent subcultures.

Remove at intervals, from the dorsal lymph sac a few drops of fluid with a sterile hypodermic syringe, after wiping skin with 70 per cent alcohol and a small spot of tincture of iodine. Inoculate an agar slant or plate with this fluid and make smears. Fix smears in mixture of alcohol and ether, equal parts, and stain with methylene-blue (Loeffler's) or toluidin. Such cultures should be made at six hours, twenty-four hours, and forty-eight hours after inoculation. If any of the animals die, make cultures and smears from heart blood and lymph sac. Use the precautions against infection that have already been insisted upon.

¹ See Metchnikoff, Immunity in Infective Diseases (English Translation), p. 137.

C. EXPERIMENTAL PATHOLOGY

Inflammation

Experiment 1. Formation of serous exudate.—Ear of a rabbit placed in water at 56 degrees C for three minutes. Examine just after exposure and at end of twenty-four hours.

Experiment 2. Purulent exudate.—Rub drop of equal parts olive and croton-oil on ear of rabbit. Examine after twenty-four hours. Examine above histologically.

Experiment 3. Sero-purulent exudate in serous cavity.—Inject suspension of aleuronat (5 c.c.) in plural cavity. Examine microscopically after forty-eight hours. (See Hiss & Zinsser).

Experiment 4. Fibrino-purulent exudate in serous cavity.—Inject 1 c.c. turpentine into pleural cavity. Examine microscopically after forty-eight hours.

Experiment 5. Repair of aseptic wound.—Carefully shave, wash and sterilize skin over an area of back of rabbit. Make an incision across muscle. See that bleeding is stopped. Place a suture of silk and apply collodion dressing.

Experiment 6. Repair of infected wound.—Repeat above procedure and purposely infect the wound with B. coli. Examine progress of wound from day to day and kill animals and examine lesions histologically after three days.

Experiment 7. Growth of epithelium over raw surface.—Prepare rabbit as in Experiment 5. Remove piece of skin. Cover with boric ointment. Examine daily the progress of epithelial growth and the formation of granulation tissue within epithelial border. Make histological preparations after three days.

NOTE: In connection with these experiments consider the experiment on the production of pyemic abscesses which will be performed next week.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Calculi, concretions, and deposits of chemical substances. Biliary calculi: their relation to inflammatory processes, their structure, and chemical constituents. Intestinal concretions. Urinary calculi—site of, chemical composition, some results of. Preputial concretions. Pancreatic calculi; Salivary calculi; Lung stones; Phleboliths; Lithopedion. Uric acid deposits in tissues, especially in kidneys; Tophi; Cholesterin deposits.

Second Day

Physical and chemical agents as causes of disease:

Trauma to embryo, during birth and *post partum*; Relation of trauma to new growths; Inanition.

Heat; cold; electricity; Roentgen rays; light;— change of atmospheric pressure; Caisson disease; metallic poisons; organic poisons; carbon monoxide; autointoxication; disturbance of function of ductless glands.

Technical Methods

Fixation; dehydration; imbedding and section cutting.

Third Day

Inflammation is a sequence of phenomena which follow injury to a part. It expresses itself by alterations in the fixed tissues including the blood vessels, by exudation of serum, by the wandering of cells derived from the blood, the predominating type depending to some extent on the character of the injury, and by proliferation and specialized activity of the cells of the fixed tissues adjacent to the injury. With it are intermingled more obviously reparative processes which restore more or less efficiently the physiological integrity of the part concerned.

The comparative pathology of inflammation. Metchnikoff's observations.

Response to injury among protozoa, slime, moulds, etc.

Response to injury among metazoa—*Daphnia* infected with monosporon.

Inflammatory phenomena observed in non-vascular areas, cornea for example.

Swelling of injured cells. Wandering in of leucocytes.

Fourth Day

Inflammatory phenomena in a vascular area—as observed in frog's mesentery.

1. Slight contraction of vessels with slowing of current in veins and capillaries. This is very transitory.

2. Dilatation of all vessels and increase of blood-flow, especially the veins.

3. Slowing of current and accumulation of leucocytes in plasma zone.

4. Attachment of leucocytes to vessel wall, especially the small veins, followed by active transmigration.

5. Diapedesis, or passive extravasation of red blood cells. The cardinal signs of inflammation: Rubor, calor, dolor, tumor.

THIRD WEEK

GENERAL OUTLINE

A. Bacteriology

Study of the cultural properties of *Staphylococci*; *M. tetragenus*; *B. pyocyaneus*; *B. melitensis*; capsulated bacilli.

B. Infection and Immunity

Second lecture on natural immunity, and one or (if necessary) two lectures on phagocytosis.

Infection of the rabbit with *M. aureus* and production of pyemic abscesses.

Study of the phenomena of phagocytosis.

C. Experimental Pathology

Continuation of experiments of last week.

D. Histopathology and Morbid Anatomy

Relations of micro-organisms to production of inflammation. Leucocytosis; types of acute inflammation. Pneumonia as illustrative of inflammation.

A. BACTERIOLOGY

First Day

Exercise 3. *Staphylococci* (a).—

Staphylococci smears are made from a pus-sample; if the material is very tenacious, dilute it with water; stain with diluted carbol fuchsin, methylene-blue and by Gram. Between the leucocytes, numerous, irregular clumps of cocci are seen, often two together, but never in chains. In the preparations, stained by Gram the bacteria seem usually to be larger than with other stains. Examine the pus in hanging drop. It is difficult to distinguish the bacteria from fat droplets, or granulations, in the inside of the leucocytes.

Pour, with one loopful, gelatin and agar plates.

Exercise 4. *Micrococcus tetragenus* (a).—

Pour, from a pure culture, agar and gelatin plates and prepare agar stab and streak, gelatin stab, broth, milk, and potato cultures. Incubate at 37 degrees and 23 degrees C. If pus containing the micrococcus is available, stain the preparations as above for *staphylococci*, and inoculate

a mouse subcutaneously. Note the capsule formation and the mode of division in hanging drop preparations.

Exercise 5. *Bacillus pyocyaneus* (a).—

The bacillus pyocyaneus is the cause of green pus. Prepare the same cultures as with the micrococcus tetragenus. Keep at 37 degrees—gelatin at 23 degrees C.

Exercise 6. *Bacillus melitensis* (a).—

Pour, from a pure culture, agar (plus 1) and gelatin plates and prepare two agar (plus 1) stab and streak, gelatin stab, broth, glucose broth, milk and potato cultures. Incubate one of each at 23 degrees and 37 degrees C, respectively.

Second Day

Exercise 3. *Staphylococci* (b).—

On the agar plates, prepared from the pus, numerous, light-yellowish colonies have developed. The superficial ones are round, those in the depth are wedge-shaped. At sixty times enlargement, the margin appears granulated. In the hanging drop, cocci are found in irregular connections to each other, mostly single and non-motile; they show molecular movements. In the stained cover-slip preparations they are always irregular and in clumps. Occasionally fission is observed. On the gelatin plates the growth is minute. At sixty times enlargement, small colonies are seen: the gelatin is in the first stage of liquefaction.

From the superficial colonies make the following subcultures: Agar, stab, agar streak, bouillon, two potatoes (23 and 37 degrees C) and gelatin stab (23 degrees C).

Exercise 4. *Micrococcus tetragenus* (b).—

On plates, round, whitish, raised colonies have developed. When touched with the needle the growth of the colonies appears to be very viscid and tenacious. The gelatin is not liquefied, the growth is abundant along the needle track. A moist growth is found in the agar and potato; the milk is not coagulated. Hanging drop and stained preparations, including Gram, are examined.

Exercise 5. *Bacillus pyocyaneus*, (b).—

The *B. pyocyaneus* produces a green-blueish pigment, which diffuses into the surrounding medium. The gelatin is liquified, the potato brownish; the bouillon is uniformly turbid and a small pellicle is formed. In the hanging drop, short, but slender bacilli, with distinct motility, are seen. Stain by Gram.

The cultures are examined for a few more days; then a stock culture (agar stab) is made and the rest discarded.

Exercise 6. *B. melitensis* (b).—

There is usually an absence of growth—or only a faint one—noticed on the various culture media. Note the results.

Exercise 7. *Capsulated Bacilli* (a).—

Inoculate a mouse with one loopful of pure culture of Friedlaender's pneumo-bacillus, subcutaneously.

Third Day

Exercise 3. *Staphylococci* (c).—

The colonies on the agar plates are darker and more orange-yellowish. They are also larger. On the gelatin plates they are now visible to the naked eye and have liquefied, perhaps, the entire plates, if the dilutions were not made carefully. At sixty times enlargement, they are roundish and granulated; the agar streak culture has a yellowish surface growth. Stab cultures are characteristic; the bouillon is profusely turbid. In gelatin stab culture, the growth is not very distinct along the lines of puncture; incipient liquefaction is noticeable. On the potato, an abundant yellowish surface growth is apparent. Litmus whey is turned pink and slightly cloudy.

The bouillon is examined in the hanging drop and in stained preparations. Instead of fixing the coverslip over the flame, keep it for two minutes in absolute alcohol. This is necessary because the substances which coagulate have, during the process of sterilization, been precipitated and removal by filtration. The bacteria would therefore not adhere to the cover-slip. Stain the preparations in the usual manner. The staphylococci appear as single or double cellular elements and not in clumps, as long as the cultures are young.

Exercise 4. *Micrococcus tetragenus* (c).—

The cultures are again examined and the result noted. A stock culture is made and the rest discarded.

Exercise 6. *B. melitensis* (c).—

A fine layer of growth is seen on the stock culture kept at 37 degrees C. On the plates, at sixty times enlargement, small colonies have developed. Prepare some stained preparations from the agar cultures. A complete examination is best postponed until tomorrow.

Fourth Day

Exercise 3. *Staphylococci* (d).—

The cultures are again examined, stock cultures made, and the rest destroyed.

Exercise 6. *B. melitensis* (d).—

Small round colonies have developed on the plates. The gelatin is not liquefied. At sixty times enlargement, one notices only the beginning of a growth. In bouillon a flocculent deposit is formed. Litmus whey has changed to a deep violet (increased alkalinity). On the potato no growth is seen. In hanging drop or stained preparations, the organism appears as a slightly motile, Gram negative, not readily stained coccus in pairs and chains. In the preparations from cultures kept at 23 degrees, occasionally bacillary forms are seen.

Keep the cultures, particularly the gelatin and milk cultures, for about two to three weeks and record the changes which occur.

B. INFECTION AND IMMUNITY

First Day

Experiments to demonstrate pyemic type of infection

Experiment 1. Two medium-sized rabbits are inoculated in the marginal vein of the ear, with different amounts of a potato culture of *Micrococcus aureus* (*staphylococcus pyrogenes aureus*). The ear is prepared by shaving the hair from the marginal vein, scrubbing vigorously with alcohol and touching a small area, through which the injection will be made, with tincture of iodine. Venous stasis can be produced, and consequent engorgement of the vein, by pressure at the root of the ear. One animal is given one loop of a twenty-four hour potato culture of the *Micrococcus* and the other animal three times as much, each contained in 1 c.c. of sterile saline solution. The injection is made by means of small sterile 2 c.c. hypodermic syringe. The following observations should be made daily on these animals: Weight, temperature, and site of inoculation. When the animals die they should be autopsied with care, as soon as possible and cultures made from the heart-blood and from any of the pyemic foci, if such be found. The lesions are to be carefully noted and smears made from them and tissue preserved in Zenker's fluid, to be cut and stained subsequently.

Second and Third Days

Experiments 2 and 3.—Demonstration to show the phagocytosis of foreign red blood corpuscles by means of macrophages, and phagocytosis of bacteria by microphages.

Two guinea-pigs are prepared for the experiment on the second day, by injecting 5 c.c. of sterile broth into the peritoneal cavity. Special directions will be given for intra-peritoneal injections. A hen or pigeon is bled by cutting the carotid artery and the blood is rapidly defibrinated and washed in sterile saline solution. This washed blood is kept in a 50 per cent suspension for the next day. A slant agar culture (entire surface) of *B. dysenteriae* (Flexner) is inoculated. Eighteen to twenty hours later the guinea-pigs are given the following injections:

Guinea-pig No. 1.—Given one-third of a twenty-four hour, slant agar culture of *B. dysenteriae* (Flexner) suspended in 2 c.c. of sterile saline.

Guinea-pig No. 2.—Given 2 c.c. of 50 per cent washed hen-blood, intraperitoneally. One and two hours later, smears are made for the entire class by withdrawing a small amount of the fluid from each peritoneal cavity by means of a capillary pipette gently inserted through the button-hole in the skin that was made for the purpose of infecting.

Smears are fixed in equal parts of alcohol and ether, or by osmic acid, and stained by methylene-blue or Giemsa stain. Make smears at subsequent periods when indicated. With these preparations may be compared those that have been made the previous week in the experiment on natural immunity of the frog.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Micro-organisms and their products the chief factors in the production of inflammation.

Portals of entry: resistance of the intact skin and mucous surfaces to infection. Lesions of skin: the hair follicles, insect bites. The nasopharynx, the nasal sinuses, the Eustachian tube and middle ear. The tonsils. The gastro-intestinal tract. The genito-urinary tract.

Locus minoris resistentiae in contrast to general susceptibility. Methods of spread of infection within the body.

Spread along mucous or other surfaces—the serous cavities.

Spread by continuity in tissues. Phlegmonous—erysipelas.

Transportation of bacteria or their toxins by lymph or blood. Lymphadenitis.

Metastatic inflammatory processes.

General reaction of the body to infection—incubation, fever, leucocytosis, reaction in the bone marrow.

Estimation of leucocytes—Differential counting.

Second Day

Technical methods.—Frozen sections: section mounting; staining.

Third Day

Types of acute inflammation,—

1. Exudative inflammation, (*a*) serous, (*b*) catharrhal, (*c*) hemorrhagic, (*d*) purulent, (*e*) fibrinous, (*f*) pseudo-membranous.

2. Alternative.

3. Productive.

The cells of acute inflammatory exudate. The serous and fibrinous content. Phagocytosis.

Pus and abscess formation.

Fourth Day

Pneumonia, illustrating a method of infection and type of acute inflammation; resistance of different individuals; and inflammatory condition in lungs. Etiology of pneumonias.

Lobar and lobular pneumonia, the former occurring in comparatively healthy individuals, the latter in less resistant persons.

Lobar pneumonia, Engorgement, the exudate in first twenty-four hours. Sero-fibrinous. Large mononuclear cells, epithelium and exudative; few leucocytes; red blood cells, more leucocytes, fibrin formation; the later stages.

Gross appearance of the two types of pneumonia; red and gray hepatization.

Association of pleurisy with pneumonia.

FOURTH WEEK

GENERAL OUTLINE

A. Bacteriology

First day.—Capsulated bacilli (continued): Pneumococcus.

Second, Third and Fourth days.—Continued study of capsulated bacilli; Pneumococcus; M. Gonorrhoea; Meningococcus.

Infection and Immunity

Lectures on opsonins and therapy of infections due to cocci.

Experiment to illustrate the leucocyte-destroying substances produced by bacteria.

Experimental Pathology

Continuation of work of second and third weeks.

Histopathology and Morbid Anatomy

Sections and experimental material illustrating: Repair following acute inflammation; chronic inflammation; granulomata; lesions due to the cocci.

BACTERIOLOGY

Exercise 7. Capsulated Bacilli (b).—

The mouse is dead; an autopsy is performed in the usual manner; the organs are examined microscopically, by smears, cultures and later from tissues fixed in formalin. In the preparations stained with fuchsin, short, plump, often oval rods, frequently two together, with a capsule, are seen. (When the culture is avirulent and the death of the animal is delayed, frequently involution forms, in shape of spheres, or globes, fine rods, etc., are seen. The staining by Gram, is positive only¹ when the decolorization is carried out with anilin xylol (two parts anilin, one part xylol). If numerous rods are present, then it is advisable to examine in the hanging drop. One observes that the rods are non-motile.

Pour gelatin and agar plates made from material from the organs.

¹ Some investigators claim that Friedlaender's Bacillus loses the stain in Gram's method, which is therefore a means of distinguishing it from Fraenkel's pneumococcus, which retains the stain in ordinary Gram's method. This holds true for only some of the strains when alcohol is used for decolorization.

Exercise 8. *Fraenkel's Pneumococcus* (a).—

If pneumonic sputum, or section of the pneumonic lung, is available, it is examined in stained preparations (diluted carbol fuchsin, Gram) and in hanging drops. One observes cocci, arranged generally in pairs (diplococci), rarely in chains of four; they are usually surrounded by a distinct capsule. The free ends of the cocci are frequently pointed and resemble a lancet; or two flames, which touch each other at the bases. Phagocytosis or intra-cellular forms are only seen after the crisis; occasionally they are entirely missing. The staining, according to the method of Gram, is positive.

Surface cultures are prepared on Loeffler's serum (three tubes inoculated with the same loop: do not flame the wire in transferring material from one tube to another), slanted glycerin agar and glycerin agar plates.

Inoculate one mouse subcutaneously, with one loopful of material.

Exercise 9. *Examination and Search for Diplococci* (a).—

If there is no pneumonic sputum available, an attempt is made to isolate the pneumococci from the saliva of healthy people. The demonstration on culture media is particularly difficult, as the very small colonies of the pneumococcus are, in a very short time, overgrown by other bacteria. For this reason, the body of the mouse is selected as a medium to obtain a pure culture; the animal is very susceptible to pneumococci but nearly immune to the bacteria of the buccal cavity. Infect four or five mice with 0.3 c.c. mixed saliva and scrapings of the tongue of several human beings, each subcutaneously.

Second Day**Exercise 7. *Capsulated bacilli* (c).—**

On the agar plates, large, round, whitish colonies have grown; when touching them with the platinum needle, short strings can be drawn off. At sixty times enlargement, they appear to be yellow-brownish, with a smooth edge.

Also, on the gelatin, the growth is fairly abundant; the colonies are raised above the surface. There is no liquefaction.

In the hanging drop, one observes plump, non-motile, short rods, in stained cover-slip preparation, a similar appearance is noticed with regard to their position; the ends are blunt, rounded; never touch each other; and are always separated by a short distance from each other; this fact is due to the development of a capsule which occurs on artificial media.

From an isolated colony agar slant, agar stab, glucose agar stab, gelatin stab, bouillon, milk and potato cultures are prepared.

Exercise 8. *Fraenkel's Pneumococcus* (b).—

The cultures prepared from the pneumonic sputum are examined; on the Loeffler's serum numerous, small, dewdrop-like colonies have developed; frequently also other colonies have developed. On the glycerin agar there is often no growth; if it is present, the colonies are even smaller than on the serum. If the slant agar cultures are examined under low power, usually a fine film is noticed. On the agar plates, at sixty times enlargement, small colonies which are transparent, with a finely granular centre and a pale, transparent periphery, are observed. In the hanging drop, non-motile cocci, mostly in pairs and short chains, are seen. Smears are prepared from the serum and impression preparations from plates. In the first preparation, aside from many normal organisms, numerous swollen ones are found. In the impression preparations, only normal pneumococci are seen.

From an isolated pneumococcus colony, gelatin stab, agar slant, agar plates, and bouillon cultures are prepared.

Exercise 9. *Examination and search for Diplococci* (b).—

If one of the mice infected yesterday has died, it is examined in the manner described in the first exercise.

Exercise 10. *Gonococcus* (a).—

From the pus of an acute case of gonorrhea, preparations are made and stained with fuchsin, methylene-blue, and by Gram, using as counter-stain safranin or fuchsin; if the pus is very viscous, the material is distributed in a drop of water. The appearance is the same as seen for the meningococci. Staining by Gram is negative. It is advisable to stain some preparations also with the following mixture: Methyl green, 0.5 grams; Pyronin .25 grams; 96 per cent alcohol, 5.0 c.c.; Glycerin, 20 c.c.; 2 per cent aqueous carbolic acid solution, add to make 100 c.c. Filter. Stain for about two minutes, rinse in water.

The student may make an attempt to cultivate the gonococcus from a fresh case of gonorrhea, in which the organisms have been demonstrated microscopically, by smearing the pus over the surface of aseptic agar-tubes.

Exercise 11. *Meningococcus* (a).—

It is supposed that the material to be examined in this exercise is obtained from a pure case of epidemic cerebro-spinal meningitis. It is an established fact that other diplococci, particularly pneumococci, may readily cause meningitis. In the smears made from the pus and stained with carbol thionin, the cocci are seen in groups of two or even tetrads, but never in chains. Two cocci usually show the adjacent margins flattened, and there is a small interval between them, which does not stain.

They resemble coffee-beans. Frequently, the cocci are found in *polymorphonuclear leucocytes*. Occasionally, cocci of a swollen appearance and some which stain poorly, are found. They do not stain by Gram's method. This feature is very important, as it permits the separation from allied diplococci, such as are usually found in the pharynx and nasal passages.

Cultures are prepared by smearing one loopful of material over agar plates, blood agar plates, Loeffler's serum or ascitic or serum-agar plates. The most satisfactory growth is obtained on ascitic or serum-maltose-agar.

For the differentiation from allied diplococci, carbohydrate media, prepared as follows, is used.

Preparation:

1. Carbohydrate (maltose, glucose, mannite, saccharose) 10.0 grams. Litmus solution—Kubel Tiemann—100.0 cc. Sterilize litmus first for fifteen minutes on a steaming water bath, then add carbohydrate and sterilize for from two to five minutes on the water bath. When cool add to each 10 ccm. of carbohydrate litmus solution .5 ccm. of normal sodium carbonate solution (sterile).

2. Ascitic fluid agar: 1 part ascitic fluid (sterile) 2 per cent chloroform for two months; 2 parts agar; to 13.5 ccm. of liquid ascitic agar add 1.5 ccm. of the litmus carbohydrate solution. Pour plates and spread micrococci to be tested on the surface of the medium.

In case no fresh material is available, cultures are prepared from a stock culture.

Smear some ascitic-agar plates with the mucous collected by means of cotton swabs from the pharynx of a healthy man to obtain meningococcus-like organisms.

Third Day

Exercise 7. *Capsulated Bacilli* (d).—

On all culture media, abundant growth is noticed. The films on the agar slant and agar stab are abundant and viscous. The growth along the gelatin stab is "nail-like." The bouillon is uniformly turbid and shows a heavy, flocculent deposit. There is a marked gas production in glucose agar. The milk is not coagulated; on the potatoes there is a moist, white, brownish layer.

The agar cultures are sealed, the other cultures can be destroyed, with exception of the gelatin culture which is examined for a few more days:

Exercise 8. *Fraenkel's Pneumococci* (c).—

In the subcultures observed yesterday, one notices an absence of growth on gelatin. On the agar, very fine grayish colonies have developed (some pneumococci strains do not develop on agar). The bouillon shows a slight turbidity. Examine, by means of a pipette, a small amount of the sediment in a hanging drop. The pneumococci form short chains.

Exercise 9. *Examination and search for Diplococci* (c).—

The dead mice are autopsied with greatest possible care, to avoid contamination. The macroscopic finding is negative; there is no pneumonia. The cause of death is a septicemia. The seat of the inoculation shows slight edema. Smears examined microscopically show the same findings as described above for the pneumonic sputum.

From the organs, agar plates are prepared and also several tubes of slanted agar and Loeffler's serum are inoculated; one loopful of blood and one piece of spleen are inoculated into a bouillon tube each.

Soak a few sterile silk threads with the mouse-blood; place them in a sterile test-tube, in which a small amount of calcium chloride, covered with cotton, has been prepared. The test-tube is sealed and kept as a stock culture. The pneumococci remain alive for a long time when dried in the manner described; whereas they die on artificial media very shortly.

It frequently happens that the mouse is infected with saliva succumbs to other bacteria than pneumococci. If, for example, short, plump rods are found only in the blood, they are examined and studied separately. In any case the experiment should then be repeated with another sample of saliva. If one finds, in the smears of the blood and the organs, indications of other bacteria, as well as diplococci, then it is highly possible that death has been caused by the latter, but the bacteria, which have been inoculated at the same time, could also develop freely in the organism reduced in vitality by the pneumococci. To obtain the pneumococci, another mouse is inoculated subcutaneously with a small piece of organ tissue; under such conditions, the saprophytes have less favorable conditions under which to multiply, than they had before.

Exercise 10. *Gonococcus* (b).—

The colonies have a diameter of 0.5 to 1 mm. They are round and gray. At sixty times enlargement, they appear to be transparent. The edges are finely granulated. In stained cover-slip preparations, the diplococci, or tetrads, are seen. To ascertain that gonococci are actually present, transplants are made from a few colonies on plain agar, on which no growth should occur.

Make transplants on serum agar and Loeffler's serum.

Exercise 11. *Meningococci* (b).—

On the ascitic and blood-agar plates, the colonies appear as circular discs, being light grayish, transparent, and having a shiny surface. At sixty times enlargement, the margins appear smooth and regular, the center slightly granulated. The colonies do not adhere to the surface of the media. On Loeffler's serum the growth is similar, if not identical. On the plain agar plates the growth is usually very poor, occasionally entirely absent. In stained cover-slip preparations, the characteristic diplococci are seen. They vary, however, in size. The Gram stain is negative.

Preparation of carbohydrate media

Dissolve 10 per cent maltose or dextrose or laevulose or other carbohydrates in litmus solution; heat for two minutes to 100 degrees C and add, when cold, to each solution 10 c.c. of 0.2 per cent normal sodium carbonate solution. Add 10 c.c. to 95 c.c. ascitic agar and pour into Petri dishes. Inoculate superficially with one loopful of culture of meningococcus.

Test the meningococci by agglutination with potent anti-serum. Use controls.

On the plates from the pharynx search for Gram-negative cocci (small colonies gray and turbid). Transplant to ascitic agar and then identify on carbohydrates.

Fourth Day**Exercise 8. *Pneumococci* (d).—**

The mouse inoculated with the sputum is examined as described previously. Particular attention is paid to the growth of the organisms in bouillon. Ascertain if only pneumococci have grown.

Add 10 c.c. ascitic agar and pour into Petri dishes. Inoculate superficially with one loopful of culture of meningococcus.

Examine sections stained by the Weigert method for pneumococci.

Exercise 9. *Examination and search for Diplococci* (d).—

The cultures prepared from the mouse are examined for the growth of the pneumococcus, as described. Prepare subcultures and inoculate bouillon particularly, to test the purity of the organisms.

Exercise 10. *Gonococcus* (c).—

The colonies on the serum agar have somewhat enlarged in size. The margin is slightly undulating. Under the microscope a fine surface growth will be noted on the slanted serum-agar tubes. The cultures can be discarded. It is not advisable to keep stock cultures, because they die out quickly.

Exercise 11. Meningococci (c).—

On the agar; light grayish, nearly transparent films have developed. On the gelatin no growth has developed. This feature serves also as distinction from allied diplococci which are Gram-negative. Only on the dextrose and maltose litmus media a red coloration has taken place. Exceptions are rarely found. The strains from the larynx are identified by a quantitative agglutination test.

B. INFECTION AND IMMUNITY**First and Second Days**

Experiment to demonstrate the leucocyte-killing substances produced by bacteria (Leucocidin)¹.—

A fluid rich in leucocytes is produced by injecting a mixture of aleuronat (prepared according to formula of Hiss & Zinsser, *Text-book of Bacteriology*, p. 290, and then diluted four or five times), or peptone solution with aseptic precautions, into the pleural cavity of rabbits or the peritoneal cavity of guinea-pigs. Both methods should be tried, but the latter will probably give the better results.

(a) The chest-wall of the rabbit is prepared by shaving, and rubbing with 70 per cent alcohol, and about 15 c.c. of the diluted aleuronat mixture is injected with aseptic precautions. On the following day the animal is chloroformed, the thorax opened, and the exudate quickly removed in sterile Locke's solution containing 0.5 per cent sodium citrate, to prevent coagulation.

(b) A guinea-pig is given 8 c.c. of the same dilute aleuronat mixture in the peritoneal cavity, chloroformed after twenty-four hours, and exudate removed as before mentioned.

Preparations of Leucocidin: 5 c.c. of a twenty-four hour bouillon culture of *Micrococcus aureus* is injected into the pleural cavity. The animal is chloroformed in twenty-four hours and the exudate removed and suspended in citrated Locke's solution, equal parts. The mixture is then centrifugalized and the clear, supernatant fluid is removed.

The bioscopic test for the demonstration of leucocidin, according to Neisser and Wechsberg, is performed as follows: A solution of medicinal pure methylene-blue is prepared as follows:

Methylene blue	1 gm.
Absolute alcohol	20 c.c.
Distilled water	20 c.c.

¹ See Kraus & Levaditi, *Handbuch der Technik & Methodik der Immunitäts*, Vol. I, p. 22.

This solution is diluted with salt solution 1-50 immediately before using. Mixtures are then prepared in the following manner with either suspension of leucocytes:

Tube A contains 2 c.c. of the leucocoidin mixture + 4 c.c. of the leucocyte suspension; Tube B contains 2 c.c. of sodium citrate solution + 4 c.c. of leucocyte suspension.

To both tubes are added several drops of the methylene-blue solution sufficient to produce a distinct but not too deep bluish color, and a thin coat of fluid, sterile vaseline is super-imposed to prevent oxidation of the methylene-blue by the air. The tubes are then placed in the thermostat at 37.5 degrees C for from one and one-half to two hours. The success of the experiment depends on the fact that living leucocytes have the property of reducing or oxidizing methylene-blue. In the tube containing leucocidin, if the latter substance is active, the leucocytes will be killed and the methylene-blue will not be reduced. If the differentiation between the two tubes is obtained as outlined, after remaining for two hours in the thermostat, smears should be made from both tubes and in the tube in which the methylene-blue is not reduced the leucocytes should show evidence of disintegration. This should also be looked for in hanging-drop preparations. The mixtures may be stained by the ordinary bacterial stains or by Giemsa, and drawings made of the leucocytes in each suspension. If the results are not satisfactory after incubation, the preparations may be left over night at room temperature.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Repair following acute inflammation.

Cells present: Polymorphonuclear, neutrophils, and eosinophils; lymphocytes and plasma cells. Endothelial cells. Fibroblasts. Fate of the micro-organism.

Factors leading to the removal of inflammatory products:

Phagocytic action and activity of leucocytes and endothelial cells; Enzyme action; Fatty alterations; Role of fibroblasts, of lymphatics, and blood vessels; Organization; Formation of new vessels or vascularization; Formation of new connective tissue; Proud-flesh; Resolution in pneumonia; Organization in pneumonia.

Second Day

Repair and reaction about foreign bodies.

Repair of aseptic wounds; healing of epithelium; of skin and mucous membrane; of liver.

Healing of septic wounds; pyogenic membrane; laudable pus.

Reaction about foreign bodies; Endothelial cells; multinucleated or foreign body giant cells; Collections about cholesterol, uric acid, keratinized cells; agar-agar and suture material.

Chronic inflammation; alterative, exudative, productive.

Infectious granuloma as illustrated by miliary tubercle. Histogenesis of the tubercle.

Exudative phenomena: leucocyte, lymphocyte, plasma cell.

Alterations in fixed tissues: Endothelial cells; endothelioid cells and fibroblasts; giant cells.

Necrosis.

Vascular reaction.

Third Day

Pyogenic cocci; staphylococcus and streptococcus.

Methods of infection; frequent secondary invaders; differences in toxicity and methods of spread.

Principal lesions produced by staphylococcus; boils and carbuncles, general furunculosis; pyemia, multiple abscesses in almost any organ of the body.

Osteomyelitis: sequestrum.

Peritonitis and infection of other serous cavities.

Infection of endocardium and intima of vessels.

Streptococcus: Its local action; general septicemia.

Erysipelas; *post-partum* infection; infections of serous cavities; middle-ear disease and meningeal involvement; infection of endocardium, etc.

Relation of various coccal forms to acute rheumatism, to endocarditis; cholera and tonsillitis.

Fourth Day

Pneumococcus: Lesions other than pneumonia. Infection of serous cavities; infections of the middle ear, nasal sinuses, and meninges. Methods of spread.

Frequency of growing pneumococcus from circulating blood.

Gonococcus: Infection of the genito-urinary tract in both sexes. The local reaction produced; character of the acute discharge; spread of the infection along mucous surfaces and by blood.

Infection of deep urethra, prostate and epididymis; urethra, vagina, Bartholins glands, cervix, endometrium, tubes and ovaries. Cause of a type of postpartum infection.

Gonorrheal endocarditis and arthritis.

Gonorrheal ophthalmia and ophthalmia neonatorum.

FIFTH WEEK**GENERAL OUTLINE****A. Bacteriology**

Throughout the week study of:

1. The streptococci.
2. Methods of disinfection.
3. Cholera-like vibrios.

B. Infection and Immunity

Second lecture on the specific therapy of infections due to the coccaceae, and a lecture on the humoral aspects of immunity.

Experiments illustrating hemotoxins; the specific protective action of antistreptococcus serum and its mode of action.

C. Experimental Pathology

Experiments to illustrate thrombosis, embolism; hydropericardium and aortic stenosis.

D. Histopathology and Morbid Anatomy

Diplococcus meningitidis; thrombosis; embolism; infarction; hypertrophy; metaplasia.

First Day**A. BACTERIOLOGY****Exercise 12. Streptococci (a).—**

Streptococci are present in pus of abscesses, in erysipelas, in the intestines of tonsils in cases of scarlet fever, in tuberculous sputum, buccal cavity of healthy persons, milk of cattle, etc.

The microscopical smears from such material are stained in the same way as for staphylococci. Frequently only diplococci are seen. Cultures are made in the following way: Pour gelatin and agar or blood-serum or ascites-agar plates, and, after coagulation, smear some of the pus material, diluted in sterile water, over the surface of the plates by means of a cotton plug fastened to a piece of wire, or distribute the material uniformly by means of a "dalli" (a bent glass rod.).

Exercise 13. Disinfection (a).—

Silk filaments are cut in lengths of one centimeter, placed in Petri dishes and sterilized at 150 degrees for thirty minutes. About 120 silk pieces are necessary. At the same time, two watch-glasses and some filter paper are sterilized in Petri dishes.

Subcultures are made from anthrax bacilli and the staphylococci stock cultures.

Prepare sufficient gelatin, agar, and broth; about fifty tubes of each.

Second Day**Exercise 12. Streptococci (b).—**

On the plates inoculated with streptococci, there are, in the majority of cases, other colonies more distinctly visible than those of the streptococci. The colonies of the cocci are very small, and frequently appear as follows at sixty times enlargement:

(a) Round, colorless colonies in the middle and margin, finely granulated.

(b) They resemble an anthrax colony on agar, with radiation of filaments.

In hanging drop; non-motile groups of cocci, occasionally chains of two or three cocci, are seen. In the stained preparations, the same picture is noticed. Frequently each coccus is divided into two hemispheres, by a line of division running at right angles to axis of the chain. Make subcultures on agar, bouillon, potato, gelatin, and Loeffler's serum.

Exercise 13. Disinfection (b).—

To have the bacteria in a handy and suitable form for the resistance tests, they are best dried on silk threads. Prepare, first, a bacterial suspension of anthrax and of staphylococci. Sterile saline is introduced into the test-tube by means of a sterile pipette and the growth is thoroughly rubbed up. The suspension is drawn up into a pipette and is transferred to a sterile filter.

The filter tube is made by drawing out a test-tube. A layer of absorbent cotton should be placed on the bottom of the tube, and then some glass wool, which acts as a weight. The filter is sterilized.

Examine, microscopically, whether aggregations of bacteria are still present; if so, it should be filtered again. Place in each of the sterilized watch-glasses 2 to 3 c.c. of the bacterial suspension (examine the anthrax suspension for spores). Some thirty silk threads are immersed in each of these suspensions. As soon as they are soaked with the fluid, they

are removed singly, by means of a pair of forceps, and are dried on sterile filter paper. One silk thread of each kind is placed on an agar plate (control).

Determination of resistance against drying.—Nine silk threads of each kind are placed in Petri dishes and kept at room temperature.

Determination of resistance against heat.—The bacteria must be completely dried on silk threads; for this purpose the damp threads are placed in Petri dishes in such a manner that they do not touch each other. After labeling the dish, place it in a desiccator over night. (Keep in ice chest to prevent sporulation).

Prepare for tomorrow.—50 to 100 c.c. of water in a small flask, and 3 test-tubes with 10 c.c. water each, are sterilized, and three agar slant cultures of typhoid bacilli are prepared from stock cultures.

Third Day

Exercise 12. *Streptococci* (c).—

The growth of the streptococci is poor; in agar stab it is better along the line of puncture than on the surface; in gelatin stab only a few single, spherical colonies, without liquefaction, are seen: broth is either turbid, or a fine, flocculent or scabby deposit has developed. The growth is more abundant when working with the *streptococcus pyogenes brevis*, but the organism is rarely found in the material mentioned above. The growth is luxurious, the gelatin is liquefied, the broth cloudy.

Microscopically, short chains of three to four elements are noted.

The long chains are best demonstrated in preparations from the water of condensation of the serum tubes or from the broth (avoid unnecessary manipulation of the fluid).

For further diagnosis, inoculate blood agar (see under Hemotoxins).

Inoculate a mouse intraperitoneally with 0.2 c.c. of a well-shaken streptococcus broth culture. For the technique consult the instructor.

Exercise 13. *Disinfection* (c).—

It is advisable to read, first, the entire paragraph carefully, as several experiments can be accomplished at the same time:

The silk threads in the desiccator should be dry.

The silk threads exposed as controls on the agar plates show an abundant growth.

Determination of the resistance against drying.—As soon as the silk threads have dried for one day, one of each is taken out of the Petri dish and placed at suitable distances from each other, on an agar plate.

Determination of the resistance against heat.—First, the resistance against a temperature of 60° C should be determined. Pour four agar tubes into Petri dishes. Place three test-tubes, containing each 10 c.c.

sterile water (prepared yesterday) in a water bath which is kept at a temperature of 60° C. One tube holds a thermometer. In each tube, six silk threads of the different bacterial types are placed and at intervals of from 2 to 5, 10, 15, 30, 60, minutes and two hours, by means of a long, curved sterile platinum wire or a pair of long forceps, one silk thread is removed at a time and placed on an agar plate. Each time, after being used, the forceps or platinum wire is properly flamed. On the Petri dishes the period of exposure to heat is marked with a wax pencil or ink.

The resistance against boiling is tested as follows: In a thin-walled beaker about 200 c.c. of water is boiled, six silk threads immersed in it, and at intervals of from 1 to 2, 5, 7, 10, 15, minutes, one of these threads is taken out and placed on an agar plate. First the staphylococci and then, in a new beaker, anthrax bacilli, are tested.

Other large articles, such as clothing, linen, etc., cannot be boiled and must, therefore, be sterilized by steam. For this exercise the infected silk threads, wrapped in filter paper with a maximum thermometer, are placed in the middle of a small parcel containing a coat or a towel, which is to be sterilized. The entire parcel is placed in the steam sterilizer. A few silk threads and a piece of paper written upon with ink are placed in a book (this book will be destroyed), also an old shoe or a piece of leather is placed in the sterilizer together with the book. A thermometer inserted in the steam sterilizer will indicate the temperature surrounding the articles that are being sterilized.

To determine the temperature inside these parcels, it is advisable to have a maximum contact thermometer which will indicate when the temperature of 98° to 100° C has been reached on starch-iodine control paper.

As soon as this temperature has been obtained the sterilizer is kept at 100° for about twenty minutes. The burner is then turned off and the articles taken out for inspection. The book is spoiled, the cover demolished, but the ink has not disappeared from the written paper. The leather is hard and useless, the clothing has not suffered. The silk threads are placed on agar plates.

Dry heat is more destructive. Silk threads are again placed in clothing or, better, in cotton parcels and heated at a temperature of 100° in a hot-air sterilizer. After from 5 to 10 and 30 to 60 minutes, one of the silk threads is taken out and placed on an agar plate.

For the next day.—Prepare, in the usual manner, two agar streak cultures of staphylococci and anthrax bacilli and cultures of each on the surface of two agar plates.

Fourth Day

Exercise 12. *Streptococci* (d).—

The blood-agar plates show varying results, according to the streptococcus examined. If around the grayish streak of inoculation a broad, clear area has formed (hemolysis), the organism studied is probably a pathogenic streptococcus. Other streptococci grow in fine, dark-greenish colonies, which do or do not—or else only to a light degree—clear up the medium. The *streptococcus mucosus* is easily recognized by its viscid, moist, slimy, gray-greenish colonies. The changes in blood-broth also depend on the streptococcus: it is either nearly transparent and Burgundy-wine red, with a few flakes at the bottom, or is turbid, brownish or greenish.

The cultures are examined for a few more days. Stock culture prepared, sealed and all the other tubes discarded.

An autopsy of the dead mouse is performed as usual.

Exercise 13. *Disinfection* (d).—

1. *Determination of resistance against drying.*—On the agar plates, on which the silk threads have been placed, an abundant, poor, or no growth, is noted. Do not misinterpret accidental dirt for bacilli. If there is no growth at all, the plates are placed back in the incubator. Note finally the absence of growth after a few days. Another silk thread from each collection is placed on an agar plate.

2. *Determination of the resistance against heat.*—Of the silk threads exposed to the heat to 60° C, a positive growth is seen on the agar plates if the silk threads have been exposed only for a short time to the heat. After a long exposure a reduction of the number and, later on, a complete destruction may be noted. The anthrax spores are not influenced at all. The staphylococci were killed immediately by boiling, the anthrax spores, on the contrary, very slowly. The disinfection with steam has caused the complete death of all the micro-organisms tested. The influence of dry heat is extremely slow, as controlled by the plate cultures. All plates are returned to the incubator, as further germs frequently develop.

3. *Determination of the resistance against a chemical disinfectant.*—(a) The use of a 5 per cent “Lysol” solution. The efficiency is tested in the following manner: First, twelve agar and twelve gelatin tubes are liquefied and kept at a temperature of 40° C. At the same time, twelve bouillon tubes are kept ready. Afterwards, a solution of the disinfectant, twice the strength to be tested, is prepared (nine parts water plus one part of lysol). A few loopfuls of the staphylococcus culture are carefully distributed in a test tube, with 2 c.c. sterile water, until a visible

turbidity is seen. Equal portions of the bacterial emulsion and the disinfecting solution (prepared in the way indicated above) are carefully mixed. Immediately afterwards, with one loopful of this mixture, an agar tube, a gelatin tube and a bouillon tube are inoculated, and the first two are poured into Petri dishes. The same process is repeated at intervals at 2, 5, 10, 15, and 30 minutes. The same exercise is repeated with the anthrax bacilli culture.

(b) To test the efficiency of a disinfectant which only reduces the development properties of a micro-organism, a 1 per cent solution of mercury-bichloride is prepared. This solution is diluted 10 times, 100 times and 1000 times. Of each of these solutions 1 c.c. is placed into 9 c.c. bouillon; dilutions of 1:100, 1:10,000 and 1:100,000 are obtained. Each of these four solutions is prepared in quadruplicate and infected with a large loopful of staphylococci or anthrax bacilli. Of each of these series, one is placed in the incubator and one kept at room temperature.

(c) Gaseous chemical disinfectants:

A. *Chloroform*.—The agar plates prepared yesterday are inverted and at the bottom of the Petri dish is placed a piece of cotton soaked with chloroform. An hour after the exposure to the chemical a subculture in bouillon is made.

B. *Formalin*.—If there is a room at disposal for this exercise, formaldehyde is generated in it in the following manner:

Mix for each cubic meter of space, two kilograms of pure potassium permanganate with two litres of formalin and two litres of water (or sixteen ounces of formalin and $6\frac{3}{4}$ ounces permanganate are recommended as suitable proportions at a temperature of 60°C).

Close all the openings of the room (ventilators, windows, keyholes, etc.), with cotton soaked in mercury bichloride. In a few drawers of the room, or in suspended clothing or laboratory coats, silk threads prepared with staphylococcus are placed. If possible, place also for this exercise some silk threads in drawers which will be kept closed. After four hours open the windows and, when the formalin has evaporated, enter the room, collect the staphylococci threads, wash them in a weak solution of ammonia, then in sterile water, and place them in bouillon or on agar plates.

Prepare for the next day.—An agar slant culture of *B. typhosus*. Sterilize in a metal box several pipettes which have been plugged with cotton, to avoid the culture material being sucked over into the mouth. at the same time sterilize twelve wooden blocks 2×1 cm. sharpened at one end like a knife. The sterilization is best done in Petri dishes. Obtain some ammonium sulphide (add H_2S to ammonia).

Exercise 14. Vibrios Resembling the Cholera Organism (a).—

(a) From an agar slant culture of the *Vibrio Metchnikovi*, the following subcultures are prepared:

Gelatin plates; gelatin stab; (use gelatin of a high alkalinity, neutral or —1 to phenolphthalein; the growth takes place with greater certainty on alkaline gelatin, but is less characteristic than on ordinary gelatin—for this reason, cultures should be prepared on both kinds of gelatin); agar plates; agar stab; bouillon; two tubes of Dunham's solution; potato.

(b) In an experiment, an attempt should be made to isolate from river water, or water of several wells, some of the most common vibrios. One liter of the water to be examined is mixed with 100 c.c. of the 20 per cent peptone solution prepared the eighth day, and thoroughly shaken; the flasks containing 100 c.c. of this mixture are placed in the incubator.

B. INFECTION AND IMMUNITY**First Day**

1. *Hemotoxins*.—Virulent streptococci produce their harmful effect in part through their destruction of red blood cells. This effect may be shown in the following manner:

Blood-agar plates are prepared by adding fresh, defibrinated rabbit blood—warmed to 40° C—to melted agar in the proportion of 1 to 4. This blood-agar is inoculated in series, with dilutions from a fresh culture of virulent streptococcus. Presence of hemotoxins is detectable by a clear halo surrounding the colonies when they appear.

Second and Third Days

2. *Negative chemotaxis by virulent streptococci in vivo*.¹—Streptococci when virulent produce their harmful effects largely through their ability to repel the protecting phagocytes.

(a) Two guinea-pigs (1 and 2) are prepared by injecting 3 c.c. bouillon intraperitoneally.

(b) Two normal guinea-pigs (3 and 4) are employed without preparing the peritoneum.

Early the next day the animals are given intraperitoneal injections as follows:

Guinea-pigs 1 and 3: Each two oese of blood agar culture of virulent streptococcus suspended in 3 c.c. of saline.

Guinea-pigs 2 and 4: Each two oese of an agar culture of non-virulent streptococcus suspended in 3 c.c. of saline.

¹ Bordet, *Studies in Immunity*, pp. 13 106, 112.

² *Ibid.*, p. 104.

A small amount of fluid is withdrawn from the peritoneal cavity, by Pfeiffer's method, at intervals of 1 hour, 3 hours, and 6 hours, and several smears are made each time. These may be fixed with osmic acid or by alcohol and ether, and stained by Giemsa. Note relations of bacteria to the leucocytes. If any of the animals die, make complete autopsy, with cultures and fixation of material.

3. *Action of anti-streptococcus serum on negative chemotaxis (in vitro)*²—Aleuronat is prepared by method of Hiss and Zinsser, diluted 5 times in normal saline and 5 c.c. of the mixture injected into the peritoneal cavity of a guinea-pig. On the following day it is removed in equal parts of citrated saline or Locke's solution as before. Virulent and non-virulent cultures of *M. pyogenes* are prepared on the second day.

On the following day the cultures are each suspended in 10 c.c. of normal saline. In sterile tubes the following combinations are made:

1. Leucocyte suspension 1 c.c.
Non-virulent streptococcus suspension 1 c.c.
2. Leucocyte suspension 1 c.c.
Virulent streptococcus suspension 1 c.c.
3. Leucocyte suspension 1 c.c.
Virulent streptococcus suspension 1 c.c.
Anti-streptococcus serum 5 drops.

The tubes are placed in the incubator and, beginning with 30 minutes, smears are made on clean cover-slips, fixed and stained as before. Take other preparations at 1 hour and 2 hours. Note relations of the micro-organisms to the leucocytes.

Third Day

4. *The protective value of anti-streptococcus serum.*—Three mice are treated as follows:

1. Given subcutaneously 1 c.c. sterile saline solution.
2. Given subcutaneously 1 c.c. normal horse serum.
3. Given subcutaneously 1 c.c. antistreptococcus serum (also from a horse).

On the next day, give each animal $\frac{1}{6}$ of a culture of virulent streptococcus subcutaneously. Note course of infections and when animals die perform careful autopsies and take cultures from the heart blood.

C. EXPERIMENTAL PATHOLOGY

Experiment 1. Thrombus formation.—Expose and ligate jugular vein of rabbit aseptically. Excise and fix after 3 minutes. Sections show platelet thrombi. Make the same experiment on a segment of vein of opposite side contained between ligatures.

Experiment 2.—Expose the jugular vein, aseptically, of a rabbit. Pinch with hemostat. Ligature carotid artery. Study results after 24 hours.

Experiment 3. Bland infarction.—Inject through the carotid of a rabbit a number of tobacco seed. Study results at autopsy one week later.

Experiment 4. Air embolism.—Inject air into ear-vein of rabbit. Follow blood pressure on kymograph. Autopsy to show method of determining presence of air in heart.

Experiment 5. Fat embolism.—Inject 2 c.c. sterile olive oil in ear-vein of rabbit. Study organs. Fix in formalin solution and stain by Scharlach R. for fat, in lungs, kidney, and brain.

Experiment 6. Hyaline thrombi.—Study sections from production of hemoglobinuria (1st week).

Experiment 7. Hydropericardium.—Under artificial respiration and anesthesia by Meltzer's method, connect carotid artery to kymograph. Expose pericardium and inject normal salt solution. Note alteration of blood pressure.

Experiment 8.—Having performed preceding experiment, aortic stenosis may be produced by passing ligature about first part of aorta; watch heart and make tracings.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Diplococcus intercellularis meningitidis.—

The nares suspected of being portal of entry. Gross appearance of brain and cord. Cells of exudate in acute and more chronic cases. Extension of exudate along nerves, to middle ear, etc. Metastatic foci in lungs. Reaction of neuroglia and of nerve cells. Recovery from meningitis. Relation to hydrocephalus and hydromyelia.

Second Day

Thrombosis is a special type of intravascular clotting occurring during life. The elements which occur in a thrombus are:

Blood platelets.

Fibrin.

Leucocytes.

Red cells.

Their arrangements in white, red, and mixed thrombi.

Factors favoring thrombosis:

Mechanical: slowing of blood stream; eddies.

Role of infection.

Injuries to endothelium.

Capillary thrombosis: Blood-platelet thrombosis. Heterologous sera.

Stroma thrombosis; heated stromata.

Thrombosis from fragments of blood elements: Ricin, *B. typhosus*.

Fibrin thrombosis.

Venous, arterial, and cardiac thrombosis.

Central necrosis of thrombi; organization of thrombi.

Exercise.—Sections: Early thrombus artery (C23); varicose vein (C355); canalized thrombus (C348).

Third Day

Embolism.—An embolus is a mass of material, such as a part of a thrombus, an oil globule, an air bubble, or decidual cells, which is carried in the circulation and finally more or less completely plugs a vessel. The interference to the circulation caused by the lodged embolus may lead to infarction.

Locations of lodgement of emboli: extremities; lungs; liver; brain; heart.

Retrograde emboli. Emboli from venous to arterial circulation.

Infarction mechanism of production—hemorrhagic and anemic.

Fourth Day

Hypertrophy. Metaplasia. Heterotopia, heteroplasia. Anaplasia or reversionary metaplasia; Keloid formation.

SIXTH WEEK

GENERAL OUTLINE

A. BACTERIOLOGY

Disinfection (continued).

Cholera-like vibrios (continued).

Isolation of cholera vibrios from water and stools.

B. INFECTION AND IMMUNITY

Two lectures on the lysins, and one on the pathogenesis, diagnosis and specific therapy of cholera.

Experiments dealing with the bacteriocidal action of normal serum; Pfeiffer's phenomenon with vibrios; artificial hemolysins; production of hemolytic and bacteriolytic sera.

EXPERIMENTAL PATHOLOGY

Experimental production of aortic insufficiency; myocarditis and arterio-sclerosis.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First and Second Days

Diseases of the heart; myocardium and endocardium.

Third Day

Passive congestion.

Fourth Day

Lesions of arteries and veins.

A. BACTERIOLOGY

First Day

Exercise 13. *Disinfection* (e).—

1. *Determination of the resistance against drying.*—Same as on the last day.

2. *Determination of the resistance against heat.*—The plates are examined to see whether a retarded growth on the different silk threads has taken place.

3. *Determination of the resistance against a chemical disinfectant.*—(a) The destructive influence of lysol solution is examined, first, on the agar plates—poured immediately after the mixture; abundant growth is seen. After one minute it is considerably less and soon ceases entirely. The number of colonies on each plate is counted by means of a counting disc and noted in a table. If the time does not permit doing so, the counting is made roughly and recorded in a tabulated form.

In a similar manner the gelatin plates are examined, but the following differences will be noted: There will be an absence of growth on the gelatin plates, when there are still a few colonies noticeable on the agar plates. Furthermore, the number of colonies is greater on the agar plates. By using gelatin plates we obtain, therefore, only an indication of destruction, as there are still some living micro-organisms present. The agar plating method is, therefore, the only suitable one.

On the contrary, a growth is found in the bouillon, when the agar plates are apparently sterile. The results obtained are, therefore, more accurate, but the bouillon method does not permit a quantitative determination. At the same time, in all such cases of unexpected growth, it is to be determined whether this abnormal growth is not due to germs in the atmosphere, a point which usually can be decided on the agar plates without difficulty. In practice the test should always be made in bouillon and agar. The cultures are returned to the incubator.

(b) The mercury bichloride has acted, in proportion to its concentration, in arresting the development of the bacilli, or antiseptically. In the first bouillon tube there is no growth at all (do not mistake albumen precipitates for growth). The next tubes show an extremely poor growth. In the highest dilution the growth is unhindered (at 37° C more pronounced than at 23° C).

The absence of growth may also be due to inhibition of the bacteria. It is therefore advisable to take a small amount of the deposit in the tubes apparently remaining sterile and inoculate some fresh bouillon. If the growth takes place there, the bacteria are alive, but they have been arrested in their development by the bichloride.

(c) The chloroform has caused quick destruction of the staphylococci. The silk threads which were placed on the plates after the formalin disinfection are sterile. The threads which remained unexposed in the drawers show distinct growth. Formalin is, therefore, a good disinfectant of the surface, but does not penetrate.

Continuation of the Chemical Disinfection.—(d) In examining silk threads with dry bacteria for resistance against a chemical disinfectant, it should always be kept in mind, and the necessary precautions taken, to avoid this disinfecting substance's developing antiseptic action in the

culture media. Washing in water is not usually satisfactory and it is better to bind the disinfectant chemically (for example, formaldehyde by ammonia). Take twelve silk threads with anthrax bacilli and place them in a 1:1000 mercury bichloride solution. After 2, 5, 10, 15, 30 and 60 minutes, remove two silk threads. One is carefully washed in sterile water, the other in diluted ammonium sulphide. The mercury is precipitated (black deposit) and the silk threads can be placed on agar plates.

(e) For the disinfection of large masses, like feces or manure, etc., lime-water is used. For the experiments, the lime which is obtainable in large pieces is sprinkled in a thin-walled beaker with about half the amount of water, until it breaks into a fine powder. Afterwards, by continuous stirring, three times the original bulk of water is added. In another beaker a small amount of manure or feces is placed and mixed carefully with an equal portion of the first prepared lime-water. After two hours, with a few loopfuls, agar is inoculated and plated. A few loopfuls are also placed into bouillon.

(f) *Phenol coefficient*: Consult Bulletin No. 82, Hygienic Laboratory.

4. *Bactericidal effect of the sunlight*.—With a small loopful of typhoid culture an agar plate is poured; to effect superficial drying place for a quarter of an hour, open, in an incubator. Afterwards, when taken out, cover the bottom of the Petri dish with a piece of black paper out of which a cross or any figure has been cut. The plate is exposed to the sunlight for an hour. The rays of the sun should meet the plate vertically. Place the plate in the incubator.

Prepare for the next day an Erlenmeyer flask with 99 c.c. and a test tube with 9 c.c. of water.

Exercise 14. *Vibrios Resembling the Cholera organism (b).*—

(1) The cultures prepared yesterday with the *Vibro Metchnikovi* are first examined. The cultures are removed carefully from the incubator, to prevent the pellicles (which have formed on the liquid media) from sinking to the bottom.

On the gelatin plates, small, white points are seen with the naked eye. At sixty times' enlargement they are light, irregular and coarsely granular, as if made up of broken glass. On agar plates, the surface colonies have a peculiar, bright, gray-brownish, transparent appearance, often with a faint bluish iridescent haze.

In gelatin stab the growth is still slight; only when a large number of bacteria have been inoculated is a funnel-shaped liquefaction noticed. In the agar stab a fine film has developed. The growth is poor along the needle track. On potato, a grayish or yellowish brown, thin, transparent layer has developed. In bouillon, the growth is abundant, the fluid is tur-

bid and a scum or pellicle is formed. The same is the case for the Dunham's peptone solution. An examination for indol and nitrates is made (inasmuch as the vibrios reduce nitrates to nitrites, it is only necessary to add one c.c. of a 25 per cent solution of H_2SO_4 to the peptone solution, and reddish pink color is produced. Should this not be the case, a few drops of a 0.5 per mille of sodium nitrite solution is added).

From the peptone solution, a hanging drop, from the agar slants, stained cover-slip preparations are made. The organisms are actively motile; they do not stain by Gram.

(2) The water samples, incubated with the peptone solution, are examined by means of hanging drops and stained cover-slip preparations.

The flasks containing vibrios, which are very active, are further examined. One tube of peptone solution is inoculated with material from the surface of the flask; gelatin plates are poured; three plates containing 3 per cent agar, which has been properly dried in the incubator, are inoculated by smearing one loopful of the surface material of the flask on the first plate, distribute regularly with a bent glass rod ("Dalli"). With the same glass rod, without being heated, smear the surface of the second, and immediately afterwards the surface of the third plate.

Second Day

Exercise 13. Disinfection (f)—

1 and 2. Same as on last day.

3. *Determination of the resistance against a chemical disinfectant.*—(a), (b) and (c): the cultures are examined again and additional notes taken.

(d) On the silk threads placed in mercury bichloride which have been washed out only in water, for a short period, no growth is visible; this would make it appear as if destruction has taken place. On all the other plates on which threads were placed, which have been washed with sulphide of ammonium, a distinct growth is visible. In the first case, the destruction was therefore simulated—as the mercury bichloride has still retained an antiseptic effect when the threads were placed on the agar.

4. *Bactericidal effect of the sunlight.*—After removing the black paper with the cross or figures, it is seen that on all the plates where the sun's rays have reached the agar plate, the culture is sterile. Perhaps along the edges where the condensed water has run over, a growth may be observed on the exposed part of the plate.

5. *Disinfection of the hands.*—One hand is slightly moistened with sterile water and then one-half of it scraped with a few of the sterilized wooden blocks, which were prepared yesterday. These wooden blocks are then

smeared over agar plates and finally placed on them. Disinfect the hands according to one of the usual ways, rinse off the disinfectant with sterile water; then scrape the other half of the hand with fresh wooden blocks. If bichloride is used, sulphide of ammonium should also be used, and then sterile water, before the blocks are smeared on agar plates, as mentioned above. The plates are placed in the incubator.

Exercise 14. *Vibrios resembling the Cholera Organism (c).—*

1. On the gelatin plates, liquefaction is distinctly visible. At sixty times enlargement, one notices that the colonies are surrounded by a circular, sharply defined ring. In the middle of the liquefied portion is a brownish yellow clump, which gradually breaks up into irregular, small particles. Liquefaction is distinctly visible in gelatin stab; a small, funnel-shaped depression is noticed. The widest diameter of the funnel is not at the surface of the gelatin, but somewhat below, and resembles more a bell-shaped, or like a separatory funnel. The liquefied part is practically evaporated and the zone of liquefaction gives an appearance of an air bubble. The potato growth has a dirty brown color.

2. The gelatin and agar plates, prepared yesterday from the water samples, are carefully examined for colonies which resemble those already described. They are examined in hanging drops and in stained cover-slip preparations. Cultures are prepared on the media described under 14A 1.

Exercise 15. *Isolation of vibrio cholerae from water and stools (a).—*

Preparation of stools containing vibrios.—Mix one loopful of a peptone solution with one gram of feces, which has been diluted previous to being mixed. Water is contaminated by adding one loopful of peptone solution culture and a small amount of liquid stool to 1 liter, and carefully shaken.

A microscopic examination is made; in the stool only a few isolated vibrios, aside from numerous other bacilli, are seen. In the water, one finds no vibrios.

The stool is examined in the following manner: Six peptone solution tubes, which have been previously heated to 40°, are inoculated with one loopful of stool (in cholera-suspected stool, select the small mucus flakes). Also, a small flask containing 50 c.c. of peptone solution is inoculated with about one c.c. of stool.

Pour gelatin plates, each with one loopful of material.

Pour plates from six tubes (3 per cent cholera agar). Place the Petri dishes, when solidified, in the incubator, inverted to evaporate the condensation water. Smear, on the surface of the agar, some of the stool

on the first plate and from there, by means of the "Dalli," material from the first plate, as described above. With the three other plates similar inoculations are made.

Three plates are poured with blood-alkaline-agar, as described before. A small amount of stool is placed on the first plate and from there, by means of the "Dalli," smeared over the second and third.

To the one liter of water add 100 c.c. of the 10 per cent peptone stock solution; shake carefully; distribute in flasks, each containing 100 c.c., and incubate.

The examination of these cultures should be made after *six, twelve and twenty-four* hours.

Third Day

Exercise 13. *Disinfection (g).—*

1, 2, 3, 4: same as on previous days.

5. *Disinfection of the hands.*—On the plates which were smeared with material collected before the disinfection, more or less numerous colonies are present (hands, which are disinfected daily, are occasionally sterile). After the disinfection they remained sterile. If there are, however, some colonies present on plates which were prepared with the block after disinfection, determine whether these colonies originate from the wooden blocks or if they are atmospheric contamination. Colonies of special type are examined microscopically and by means of cultures.

Exercise 14. *Vibrios resembling the Cholera Organism (d).—*

1. On the gelatin plates, the liquefaction has progressed, and at sixty times enlargement dissolution of the colonies is very distinct. The appearance of the gelatin stab cultures is the same as yesterday—perhaps more distinct and characteristic—or the liquefaction has spread downwards towards the needle track, or has spread over the sides of the tubes.

Two agar slants are prepared to be kept for stock cultures; the other cultures are destroyed.

Exercise 15. *Isolation of Vibrio cholerae from water and stools (e).—*

On the agar plates a search is made for the colonies of the vibrios, which are readily recognized on account of their bluish iridescence. When found they are transplanted and examined further.

On the blood-alkaline-agar, the colonies are very large, round, with a smooth margin and, in transmitted light, perfectly clear.

One selects a single isolated colony and makes a transplant by touching the colony with a platinum wire under the low power. On the gelatin plates, only a few scattered colonies of the vibrios are visible. The peptone solution cultures show scums which consist entirely of vibrios when examined in a hanging drop. In the peptone solution an enrichment of the vibrios took place.

If on the agar, or blood-alkaline-agar, no pure colonies are obtained, agar plates and blood-agar are inoculated from a peptone solution tube containing numerous vibrios. In addition to these cultures, four fresh peptone solution tubes are inoculated.

(For cholera diagnosis, the blood-alkaline plates are used. The organisms of a typical colony are agglutinated with anti-cholera serum in descending dilutions; at the same time, a slanted agar is inoculated and Pfeiffer's reaction carried out the next day. If all these tests are positive, the colony examined was really a colony of the *Vibrio cholerae*.)

Fourth Day

Exercise 13. Disinfection (h).—

Similar inspection as on the other days. Complete the tabulation of the results; the plates which have been kept in the incubator for seven days can be eliminated.

Exercise 15. Isolation of *Vibrio cholerae* from water and stools (c).—

The agar and blood-alkaline-agar plates prepared from the peptone solution cultures, are examined in the same manner as yesterday.

B. INFECTION AND IMMUNITY

First and Second Days

1. Bactericidal property of normal fresh serum (Buchner's alexin).—

Fresh serum is obtained by bleeding one or two large guinea-pigs from the carotid into a specially prepared test-tube with capillary end, where it is allowed to clot. When the clot has become firm it is carefully separated from the walls of the tube by a sterile glass rod, with aseptic precautions. On the following day the serum is found to be well separated from the contracted clot. The bloody serum is centrifugalized to render it free from corpuscles, and half the serum is heated in an acetone bath at 56° for one-half hour. An agar culture of *Vibrio Metchnikovi* is prepared on the first day, and on the next day one oese is suspended in 5 c.c. of bouillon and from this tube dilution II is made in the usual way by transporting three oese from tube I.

The following combinations are then prepared in small sterile test-tubes:

Tube 1.	Fresh guinea-pig serum	0.5 c.c.
	Sterile bouillon	0.5 c.c.
	Dilution Sp. Metchnikovi	I.
Tube 2.	Guinea-pig serum, 56°	0.5 c.c.
	Sterile bouillon	0.5 c.c.
	Dilution Sp. Metchnikovi	I.
Tube 3.	Sterile bouillon	1 c.c.
	Dilution Sp. Metchnikovi	I.

Tubes 4, 5, 6, identical in composition with 1, 2, 3, but with dilution II of *Sp. Metchnikovi*, replacing Dilution I.

Allow these tubes to stand in the incubator for from three to four hours and then plate in agar, using the entire contents of each tube.

Count number of colonies in twenty-four hours after incubation at 37.5° C.

Second and Third Days

2. *Pfeiffer's phenomenon in vitro (Bordet's method)*.¹—Fresh alexic serum is prepared from a guinea-pig, as in the last experiment, and part of it heated to 56° C as before. Both sera are diluted 1–10 in saline, for use as designated below. An entire surface culture of *sp. cholerae* is also inoculated and on the next day suspended in 5 c.c. of sterile saline. The 5 c.c. are further diluted with 1 to 10. An antiserum prepared by immunizing rabbits with cultures of *Vibrio cholerae* (Serum rabbit *Vibrio cholerae* 56°) and inactivated at 56° will be provided. The method of preparing such antisera is described below and will be carried out by members of the class.

A suspension of another vibrio (e.g. *Sp. cholerae*) should also be prepared as with *Vibrio Metchnikovi*.

The following tubes are prepared:

1. Suspension *Sp. cholerae* 1 c.c.+ fresh diluted guinea-pig serum 1 c.c.+ normal saline 1 c.c.
2. Suspension *Sp. cholerae* 1 c.c.+ fresh serum guinea-pig 1 c.c.+ S. rabbit-anticholera 1–50, 1 c.c.
3. Suspension *Sp. cholerae* 1 c.c.+ Serum guinea-pig 56° 1 c.c.+ S. rabbit anticholera 56° 1–50, 1 c.c.
4. Suspension *Sp. cholerae* 1 c.c.+ fresh guinea-pig serum + S. normal rabbit, 56° 1–50, 1 c.c.
5. Suspension *Sp. Metchnikovi* 1 c.c.+ fresh guinea-pig serum + S. rabbit-anticholera 56°, 1–50, 1 c.c.

Allow these tubes to stand in incubator for 30 minutes and then make smears, fixing in the usual manner, and staining with toluidin blue or carbol fuchsin (dilute). If results are not satisfactory, make smears at later intervals. Note and draw condition of vibrios.

Third and Fourth Days

3. *Artificial hemolysins*.¹—Alexic and heated guinea-pig serum is prepared as in previous experiments. Washed sheep red blood corpuscles are obtained by bleeding a sheep, through a trocar, from the jugular vein and catching from 15 to 25 c.c. of blood in a sterile flask containing

¹ Bordet, *Studies in Immunity*, p. 56.

glass beads, where it is defibrinated by agitation. About 5 c.c. of the defibrinated blood is placed in a large centrifuge tube and the level of the whole blood is marked on the outside of the tube with a wax pencil. The tube is then nearly filled by adding 40 c.c. of normal saline and shaken. The tube is centrifugalized, the supernatant fluid removed, and fresh saline added. After centrifugalization, the operation is repeated a third time. After removing the last wash-water the original volume of the blood is restored by adding saline. These red blood cells, washed free of serum, are usually used in a 5 per cent suspension in saline for hemolytic experiments. Washed guinea-pig blood is prepared in a similar manner from the blood left after centrifugalizing the alexin. The other material employed will be furnished, namely: a rabbit-anti-sheep hemolytic serum (56°), and normal rabbit serum.

The following tubes are prepared by the class:

1. Washed sheep corpuscles 5 per cent 1 c.c.+ normal rabbit serum 56° 0.01 c.c.²+ alexin of guinea-pig 0.05 c.c.
2. Washed sheep corpuscles 5 per cent 1 c.c.+ rabbit-anti-sheep serum 56°, 0.01 c.c.+ alexin 0.05 c.c.
3. Washed sheep corpuscles 5 per cent 1 c.c.+ rabbit-anti-sheep serum, 56°, 0.01 c.c.+ alexin 0.05 c.c.
4. Washed guinea-pig corpuscles 5 per cent 1 c.c.+ rabbit-anti-sheep serum, 56°, 0.01 c.c.+ alexin 0.05 c.c.

These tubes are left at room temperature or immersed in water at 37°, and hemolysis watched for. In these dilutions it should occur in a few minutes.

The minimal hemolytic dose (M.H.D.) or hemolytic titre of the immune serum employed may be determined in the following manner: Such a determination is necessary in many of the practical uses to which this test is put. Starting with the dilution containing 0.01 c.c. of the heated hemolytic serum in 1 c.c. of saline, the serum is successively diluted in equal amounts of saline placed in separate tubes. Thus dilutions of 0.005, 0.0025, are readily obtained. A good hemolytic serum readily produces complete hemolysis in a dilution of 0.001 or beyond. The dose just sufficient to produce complete hemolysis is the minimal hemolytic dose. To each such dilution the regular doses of washed blood cells and alexin are added.

4. *Production of bacteriolytic and hemolytic sera.*—Rabbits are the best animals to use for the production of these antisera. The injections may be given subcutaneously, intraperitoneally or intravenously, and the intervals between injections may be from one to ten days. The size of the

¹ Bordet, *Studies in Immunity*, p. 134.

² Diluted in 1 c.c. of saline solution.

dose depends on the route of injection chosen and the toxicity of the alien protein, particularly in the case of bacteria. The rapid and intensive method is best when it can be employed, as it yields results in the shortest time. The following immunizations will be carried out, each by two students:

1. Immunize two rabbits with 1 c.c. of washed sheep blood injected intravenously on three successive days. Test hemolytic titre of serum on fifth and tenth days after last injection. Bleed animals to death and keep serum.

2. Immunize two guinea-pigs with three intraperitoneal injections of 2 c.c. defibrinated washed rabbit blood at three-day intervals. Bleed to death ten days later and test hemolytic titre. (Use G.P. alexin).

3. Immunize two rabbits with four intravenous injections on successive days, each of a 1/10th of a killed (60 degrees) agar culture of *Vibrio cholerae*, or *B. typhosus*, or *B. paratyphosus* B and A, or *Meningococcus*. Test for bacteriolysins (Pfeiffer's phenomenon) and agglutinins and opsonins ten days after first injection.

4. Immunize 5 guinea-pigs by injecting them with a killed culture of *M. pyogenes aureus*. Heat a 24-hour agar culture in 0.85 NaCl solution 50° C for 30 minutes. Begin injections with 1/50 24-hour culture, give 4 injections ending with 1/10 24-hour culture. Total volume of fluid injected should be 2 c.c. and the injection should be given intraperitoneally. The blood serum of these animals will be used in experiments on opsonins.

5. Immunize two rabbits to fresh cow's milk by giving them increasing amounts by intraperitoneal injection. Begin injections with 2 c.c. and increase to 5 c.c. and 10 c.c. as soon as the weight of the animal permits. The blood serum of these animals will be used in work on precipitins.

6. Immunize four 300-gram guinea-pigs to a solution of egg-white in 0.85 per cent NaCl solution. The solution of egg-white should be 1 to 10. Give injections of 2 c.c., 3 c.c., 5 c.c., and 10 c.c. every three to four days by intraperitoneal method. The blood serum of these animals will be used in experiments on precipitins.

7. Inject four 300-gram guinea-pigs with a solution of egg-white as described above, giving one dose of 1 c.c. subcutaneously. These animals will be used in experiments dealing with hypersusceptibility, or anaphylaxis.

8. Give four 300-gram guinea-pigs a single injection of 0.1 c.c. each normal horse serum subcutaneously. These animals are to be used for experiments in anaphylaxis later in the course.

EXPERIMENTAL PATHOLOGY

Experiment 1. Aortic insufficiency.—Meltzer's method of anesthesia. Pass valve hook through carotid artery of rabbit and rupture the aortic valve. Make tracing of the resultant lesion.

Experiment 2. Myocardial lesion.—Meltzer's method of anesthesia. Open pericardium of dog and ligate (using round needle) a branch of the coronary artery. Note alterations of heart's action. Record the results on a kymograph.

Experiment 3. Arterio-sclerosis.—Inject adrenalin solution into ear-vein of a young rabbit, beginning with 3 drops and increasing the dose, by one drop daily, for one week. After 2 weeks, make autopsy and study the aorta and cardiac muscles grossly and on section.

D. MORBID ANATOMY AND HISTOPATHOLOGY

First Day

Heart.—

Congenital malformations: Foramen ovale; perforate or absent interventricular septum; abnormal cordae tendinae; abnormal valve segments—fenestration of.

Congenital hypoplasia or hyperplasia.

His's bundle.

Brown atrophy.

Fatty infiltration and excessive epicardial fat.

Fatty degeneration.

Vacuolar degeneration (hydropic).

Segmentation and fragmentation.

Cardio Malacia.

Other degenerations.

Endocarditis.—acute-verrucous and ulcerative. Thrombi-endocarditis. Valvular and mural.

Relation to tonsillitis, rheumatic fever, chorea, and other infectious diseases.

Frequency of attack on various valves.

Relation to formation of cardiac aneurism.

Myocarditis.—

Parenchymatous—cloudy swelling—Diphtheria.

Purulent.

Interstitial and rheumatic.

Acute pericarditis.

Second Day*Chronic endocarditis.*

Recurrent endocarditis.

Chronic endocarditis. Of inflammatory origin.

Valvular—Mural.

Of arterio-sclerotic origin.

Of toxic origin.

Chronic myocarditis and coronary sclerosis—angina pectoris.

Stokes-Adam syndrome.

Cardiac hypertrophy—its cause and significance.

Arising outside the heart.

Arising within the heart.

Metallic poisons—exophthalmic goitre.

Relation of cardiac vascular and renal disease.

Chronic pericarditis.

Primary and metastatic new growth of the heart.

Third Day

Chronic passive congestion of cardiac origin.

Valvular insufficiency—organic and functional.

Cardiac insufficiency—cardiac dilatation.

Passive congestion—general considerations.

Pulmonary; hepatic; Splenic.

Anasarca.

Fourth Day

Lesions of arteries and veins.

Acute lesions of arteries and veins.

Rupture.

Inflammations.

Endarteritis of syphilitic origin (Heubner's endarteritis).

Endarteritis obliterans (arterio-capillary fibrosis).

Periarteritis nodosa.

Hypertrophy of vessels.

Experimental production of arteriosclerosis.

Adrenalin, barium salts, changes in blood pressure.

Etiology of arterio-sclerosis.

Inherited predisposition.

Toxic including bacterial toxins, heavy metal, etc.

Atheroma and arteriosclerosis of aorta.

Syphilitic aortitis.

Senile changes in arteries.

Von Monckeberg's form of arterio-sclerosis.

Calcospherites of Pick.

Varices.

SEVENTH WEEK

GENERAL OUTLINE

Bacteriology.—Study of *B. typhosus*, *B. coli*, and *B. enteritidis*.

Water analysis.

Isolation and identification of bacteria.

Infection and Immunity.—Lectures on tropins, and on agglutination.

Study of the action of hemotoxic serum and of the phenomena of agglutination.

Histopathology and Morbid Anatomy.

First day.—Aneurysms.

Second day.—Hematopoietic system and its diseases; Leukemia.

Third day.—Anemias.

Fourth day.—Diseases of the cardio-vascular system.

A. BACTERIOLOGY

First Day

Exercise 16. *B. typhosus* and *B. coli* (a).—

Pour gelatin plates from a pure culture of *Bacillus typhosus* which is obtained from the laboratory. Take only a very small quantity with the platinum needle (nothing should be seen on the needle).

Pour gelatin plates from normal feces (three platinum loopfuls of feces in the first tube). The feces are examined at the same time by means of smears, and stained in the usual manner. Usually only rod-shaped bacilli are seen.

Exercise 17. *Water analysis* (a).—

A bacteriological analysis of water consists: (1) in the determination of the number of bacteria; (2) in the identification of the several species; (3) the recognition of the pathogenic bacteria present. The standard methods for the examination of water, published by the American Public Health Association, Second edition, 1913, are followed.

Prepare and keep on hand about 20 tubes nutrient gelatin (10 per cent); agar (1 per cent) (reaction + 1); lactose-litmus agar; liver broth; lactose bile.

1. *Lactose-litmus agar* is prepared in the same manner as nutrient agar; just before sterilization, 1 per cent lactose is added to the medium and sterilized in an autoclave at 120° C for fifteen minutes only. An aqueous solution of Kahlbaum's azolitmin (boiled for five minutes to dissolve) is sterilized separately.

2. *Liver broth*.—Chop 500 grams of beef liver and add 1000 c.c. of distilled water; boil slowly for two hours in double boiler, starting cold and stirring occasionally. Make up loss by evaporation; pass through wire strainer. Add to filtrate 10 grams peptone, 10 grams glucose and 1 gram of potassium phosphate. After warming this mixture in a double boiler and stirring it for a few minutes to dissolve the ingredients, titrate with N/20 NaOH. Boil vigorously for 30 minutes. Make up loss by evaporation, filter through cotton and filter paper. Tube and sterilize in an autoclave for fifteen minutes, at 120° C. The reaction should be + 1.

3. *Lactose-bile medium*.—Sterilized, undiluted ox gall, with 1 per cent peptone and 1 per cent lactose are sterilized in Smith tubes.

Sterilize three bottles with glass stoppers (250 c.c. capacity) at 160° C for one hour, in the hot-air sterilizer. Sterilize 10 c.c. pipettes divided into 1/10, and 5 c.c. pipettes, wrapped in paper or a towel. Sterilize 6 test-tubes with 9 c.c. distilled water.

Exercise 18. *B. enteritidis* (Gaertner) (a).—

A mouse, of which the feces have been examined and found to be free from para-typhoid organisms, is fed with *B. enteritidis*; an agar slant prepared yesterday is washed off with a small amount of water and in this is soaked a small piece of bread. This material is fed to the mouse. Symptoms may be expected in a few days.

A quicker result can be obtained by inoculating another mouse subcutaneously, with one loopful of material; the death usually occurs after one or two days.

Second Day

Exercise 16. *B. typhosus* and *B. coli* (b).—

On the first typhoid gelatin plates there are a great number of small gray spots; at sixty times enlargement the superficial colonies are fine and transparent, with a smooth margin. From the centre, small lines run towards the margin. The small colonies in the depths are yellowish and round. No chains are observed. Prepare impression preparations from two small colonies. Several preparations are stained in the usual way. Stain also by Gram. Make subcultures on slant agar. Colonies are selected under the microscope for this work.

On the plates which have been poured from the feces, colonies similar to the typhosus colonies are seen. Several are examined microscopically and sub-cultured on slant agar and incubated.

Exercise 17. Water analysis (b).—

Examine ordinary spigot water; polluted well or river water. The spigot water is obtained in the following way. Let a few drops of water run through the spigot, then flame the opening with a Bunsen burner. As soon as the steam appears, let the water run for about 20 minutes; flame the neck of the sterilized bottle, and fill it. The examination should take place not more than half an hour after the collection.

The procedure of plating is as follows:

1. Shake the bottle at least 25 times. Withdraw 1 c.c., 0.5, and 0.1 c.c. with a sterilized pipette and deliver it into sterilized Petri dishes (10 c.c. diameter). To the liquid in the dish add 10 c.c. of standard agar at a temperature of about 49° C. Mix the medium and water thoroughly by tipping the dish back and forth, and spread the contents uniformly over the bottom of the plate. Allow the agar to cool rapidly on a horizontal surface and transfer to 37° C incubator as soon as it is hard.

2. To collect water from a well, complicated methods are usually necessary. For our purposes, a bottle on a string or wire is lowered into the well and the collected sample is used for examination.

Proceed, as explained above, using the following quantities: 1.0, 0.1, 0.01, 0.001 and 0.0001 c.c. of water. These dilutions are necessary when one suspects that the highest dilution will show more than 200 colonies per c.c. The dilutions are best made as follows: Shake the sample twenty-five times and mix 1 c.c. with 9 c.c. of sterilized water, shake carefully, and measure 1 c.c. of the diluted sample with a fresh, sterile pipette into a Petri dish. The higher dilutions are prepared by mixing 1 c.c. of the diluted sample with 9 c.c. of water and proceeding as usual. Pour these plates in duplicate together, with one set of gelatin plates prepared with the same dilutions, using, instead of agar, the above-mentioned medium. Always use fresh, sterilized pipettes for each dilution.

Pour one agar and one gelatin plate to control the sterility of the media, one with the water sterilized for the dilutions, one for testing the pipettes, etc. The gelatin plates are incubated for five days at 20°.

Exercise 16. *B. typhosus* and *B. coli* (c).—

1. The colonies on the gelatin plates are larger. The *coli* colonies are larger than the *typhosus* colonies. The structure on the inside of the superficial colonies has disappeared. From these colonies, preparations are made and stained as described for anthrax. In the center an "S" or loop-shaped structure is seen; the margin is made of long rods and filaments which return to the center, giving the colony its lobulated character.

2. On the agar streak cultures, a grayish deposit is formed, which is thicker in the *coli* than in the *typhosus* tubes. In the hanging drop, small bacilli with very active movements are seen. In the stained preparations they appear somewhat larger when made from gelatin, than from agar cultures. They do not stain by Gram, and take, when restained, the red stain with carbol fuchsin.

3. Three agar plates are made from *B. typhosus* and *B. coli* cultures. Then sub-cultures are made on agar streak, on agar stab, gelatin streak, gelatin stab, potato, milk, litmus-whey and fermentation tubes (to be supplied by the laboratory). At the same time, *shake cultures* in glucose agar are made in the following way: One liquefied and cooled tube is inoculated with a loopful of *typhosus*, one with *coli* bacilli. Carefully mix the agar, coagulate the tube, standing upright, in ice-water. Label and incubate these cultures. At the same time, four tubes containing sugar agar are liquefied, and one drop of a sterile neutral red solution is added. Boil the tubes for five minutes, cool them off and inoculate them in the same way as just described. Coagulate the agar and label as described.

Two bouillon tubes are inoculated with *B. typhosus*, and from three *B. coli* cultures, subcultures in two bouillon tubes each are made and incubated.

Exercise 17. Water analysis (c).—

The colonies on the agar plates are counted. If only a small number of colonies are present, they can be counted with the unaided eye, but when—as it frequently happens—the number is very large, it is desirable to make use of a counting apparatus. Place the Petri dish on a glass plate suitably ruled and count the colonies with the aid of a lens which magnifies at least five diameters. Express the results according to the standard methods.

The gelatin plates are also inspected; the colonies are innumerable, but the liquefying species of bacteria are transplanted on agar slants; studied microscopically and by means of cultural tests. The modified Chester chart is used to advantage.

To avoid spreading of the liquefying colonies, they are best destroyed after transplants have been made. By means of a pair of forceps, add a few crystals of potassium permanganate; the crystals will dissolve and the disinfecting action will spread and interfere with the growth of the other colonies.

GENERAL HINTS FOR IDENTIFICATION

The tests for differentiating bacteria are usually divided into two groups—primary tests and supplementary tests. The first are of chief importance: they include those tests in which a definite positive or

negative result may serve as a basis of a classification. The supplementary data are of less importance, but are desirable.

Preliminary Cultivation.—Recent growths of the bacteria are necessary to obtain greater uniformity in the cultural characteristics. Make successive transplants into broth, always incubating twenty-four hours at 20° C. From the third broth-culture make a gelatin plate and incubate for forty-eight hours at 20° C.

From one of the colonies on the gelatin plate inoculate a tube of slanted agar, incubate for forty-eight hours at 20° C, and use it for making subsequent inoculations in the various media.

The morphological characters are determined from at least one solid and one liquid medium incubated at 37° C for twenty-four hours, and at 20° C for forty-eight to seventy-two hours. The growth is examined in stained preparations and in hanging drops. Morphological descriptions should always be accompanied by notes regarding the medium used, the temperature and age of the culture. Determine: forms and grouping, dimensions (stained by aqueous gentian violet), unstained internal structure in stained preparations (watery dye, anilin gentian violet; Gram's stain) capsules, spores, flagella, involution forms and fission (hanging drop method).

The cultural characters are determined by noting, after a certain period of incubation: (1) the mass characteristic of the organisms and (2) the biochemical reaction they produce.

1. The following data are required (Standard methods):

(a) *Nutrient broth.*—

Condition of fluid, whether clear or turbid; character of turbidity; amount of sediment, etc.; surface pellicle, color, consistency, structure; reaction and odor.

(b) *Gelatin or Agar plates.*—

Surface colonies: Form of colony; size of colony; surface elevation; topography of surface; microscopic structure of margin of colony; color, determined both by transmitted and reflected light; luster, consistency.

Deep colonies: Form; size; microscopic structure; consistency; color; change in surrounding media.

(c) *Gelatin or Agar Tubes.*—

Stab cultures: Growth along line of puncture; surface growth (same as for plate cultures); extent of liquefaction, if any.

Streak cultures: Form; size; surface elevation; topography of surface; color; consistency; change in medium.

The period, temperature and general environmental conditions during cultivation should be noted. Generally, the incubation at 20° is four days or for the determination of liquefaction fourteen days; at 37° it is 48 hours.

2. For the chemical study, the following requirements are essential:

(a) *Milk*.—

Incubate at 37° C for 48 hours; observe the time required to curdle; the character of the curd; whey; the digestion of the casein; production of gas and the odor. In case no curd is formed, boil the milk for a few seconds and note if curdling follows. No. 2. Do not use azolitmin as an indicator in the medium alone, but use the titration method.

(b) Action on carbohydrates lactose, glucose, saccharose, etc., is best studied in fermentation tubes. Heat the tubes in the sterilizer before inoculating; when cool, inoculate the surface of the liquid in the bulb with the culture to be tested. Incubate at 37° C for 48 hours or longer. Observe from day to day, with the aid of a gasometer, the amount of gas produced.

At the end of incubation remove the tubes from the incubator and allow the liquid to come to room temperature. Express the amount of gas in per cent.

(c) *Action upon nitrates*.—

Inoculate a tube of nitrate broth and incubate for four days at 37° C, together with an uninoculated tube, to serve as a blank for comparison. At the end of that period, remove 3 c.c. of the culture to a clean test-tube and add 2 c.c. of each of naphthylamin solution and the sulphanilic acid solution.¹ The red color indicates the presence of nitrites.

4 c.c. of the above-mentioned culture is placed in a second test-tube and tested for the presence of ammonia, by adding a few drops of Nessler's solution.

(d) *Production of indol*.—

Inoculate broth and incubate for four days at 37°. At the end of that time, add 1 c.c. of 10 per cent sulphuric acid and mix the solutions by shaking. Then carefully allow to run, on top, 1 c.c. of 0.01 per cent solution of freshly made sodium nitrite and allow to stand for one-half hour or more. A pink ring indicates the presence of indol. A blank determination should always be carried on.

¹ A-amido naphthalen acetate solution. Dissolve 5 grams of solid A-naphthylamin in 1000 c.c. of 5 N acetic acid; filter through absorbent cotton. (B) Sulphanilic acid solution: Dissolve 8 grams of the purest sulphanilic acid in 1000 c.c. of 5 N acetic acid (Sp. Gr. 1.041).

Ehrlich's roseindol reaction is successfully used. The method is described on page 85 of Muir & Ritchie, Sixth Edition.

(e) *Relation to Oxygen.*—

This determination is best made by growing the organisms with free access of the atmosphere, or with complete exclusion of the air, by one of the recognized methods of anaerobic cultures.

(f) *Temperature relation.*—

Determine the growth at 20° C and at 37° C and the extreme limits within which development occurs. The most favorable temperature for development and the thermal death-point of the bacteria, both in the vegetative and in the spore state, should be investigated.

(g) *Pigment formation.*—

Grow the organisms in presence or absence of oxygen, on various media, at 20° C and 37° C.

Among the supplementary tests, the following are briefly mentioned:

Cultures on potato, or blood serum, and at times various carbohydrates. It is important occasionally to test the pathogenicity by inoculating various animals.

It is advisable to select one or two of the numerous colonies on the plates, and carry them through the tests, using the tabulated results given by Fuller, *Journal of Experimental Medicine*, vol. 4, 1899. For identification, use Lehmann and Neumann, *Atlas und Grundriss der Bakteriologie*, 5te. Auflage, 1912.

Fourth Day

Exercise 16. *B. typhosus* and *B. coli* (d).—

The colonies on the gelatin plates are unchanged. On the agar plates the superficial colonies are gray and wedge-shaped. The *B. coli* colonies are larger than those of the typhoid. At sixty times enlargement, nothing characteristic is seen.

In the stained impression preparations, the bacilli are mingled irregularly. In hanging drop they are very motile and cannot be distinguished from the typhoid bacilli. The bouillon cultures are turbid. The gelatin and streak cultures show a slight, iridescent film. The medium is not liquefied. On the agar streak nothing characteristic is seen.

The milk, inoculated with the typhoid bacilli, shows no change. The milk inoculated with the colon bacilli is coagulated.

On the potato culture, the *B. coli* shows a yellow-brownish film. In the culture of *B. typhosus*, it will seem, at first glance, as if nothing has grown. Only when examined by means of smears, which are made in the usual way, it will be noted that the bacilli have increased and form

an invisible growth. The *B. typhosus*, in the stained preparations from potatoes, often shows unstained patches which have no relation to spore formation. In such bacilli the protoplasm has contracted from the edges, and only this part of the bacillus is actually stained. This process is called "plasmolysis."

In agar, *B. typhosus* has only caused cloudiness. The colon bacillus has produced large gas bubbles which have split the agar, and in the neutral red tubes the stain has been reduced. Therefore, the cultures appear, in transparent light, reddish; and in refracted light, greenish and fluorescent.

Should this color not appear very distinctly, the agar is liquefied and a small amount of alkali is added. Unreduced neutral red appears orange—reduced, greenish and fluorescent.

In the litmus whey, *B. typhosus* shows practically no growth. The tubes appear slightly reddish; therefore a small amount of acid has been produced. The colon bacillus produced a large amount of acid, the litmus whey is bright red and distinctly turbid.

In the fermentation tubes, *B. coli* has produced a considerable amount of gas. *B. typhosus* has only caused a turbidity.

Exercise 17. Water analysis (d).—

The agar and gelatin plates prepared, are examined and counted, as explained before.

The examination of some of the organisms is continued.

Determination of B. Coli.—The typical *B. coli* is found everywhere in large quantities where dejecta of human beings are present. From the quantity of the coli present deductions can be made on the degree of pollution of a given water. This feature is particularly important in water analysis. The methods used for the determination are usually divided into qualitative and quantitative tests. They are usually combined.

Procedure.—Inoculate fermentation tubes of lactose bile, with the following quantities of water collected from a suspected well: 0.1, 1, 5, 10, and 20 c.c.; incubate at 40° C for 24 hours; if gas appears, a portion of the liquid is plated on litmus-lactose agar, for the determination of *B. coli*. All the colonies which are red on these plates can be regarded as *B. coli*, but should always be tested by inoculating various carbohydrate solutions. For this identification, the methods given by the American Public Health Association are best. This procedure has quantitative value only when numerous portions of the water sample are tested to ascertain the approximate volume above which the results are positive, and below which they are negative.

Another method, which can be recommended, is the following: 100, 50, 15, 5, 0.5 c.c. of water are mixed with liver broth; in the first dilutions, in equal parts; in the latter, with 10 c.c. each. If the sample is very polluted, it is best to dilute in the following manner: 0.5 c.c. water is diluted with 50 c.c. and the same process repeated a third time. From each dilution, inoculate 1 and 0.1 c.c. into 10 c.c. bouillon; incubate at 37°. After 24 hours, some samples are uniformly turbid; some are turbid only on the top, some at the bottom only. Some remain entirely clear. Only the first samples are further examined, by inoculating litmus-lactose agar plates and by examining the red colonies as usual.

Should the inoculated broth give off a strong odor, a test for *B. sporogenes* should be made, as follows: The contents of the tubes are poured into separate, sterile, Erlenmeyer flasks or large test-tubes and heated at 80° C for 10 minutes. One c.c. of broth is withdrawn from the bottom of each flask, or tube, and is planted separately into a second set of sterile liver-broth fermentation tubes and incubated for 24 hours, after which time gas formation and a characteristic odor will again be observed. The microscopic examination will reveal numerous, large, sluggishly motile bacilli containing spores. For further identification test, consult the standard methods.

Exercise 19. *Isolation of the typhoid bacilli from the stools and the water*
(a).—

Preparation of the media

1. *Litmus, nitrosc-lactose agar, according to V. Drigalski-Conradi.*—

(a) 0.75 kilogram of horse-meat is treated with two liters of water over night. Prepare further as described later.

2. (a) Prepare one liter of neutral (+ 0.2) 3 per cent agar: 1000 c.c. water, 30 grams agar; 10 grams peptone; 10 grams Liebig's meat extract; 5 grams NaCl. This mixture is boiled for two hours at 110° C. Filter through cotton, distribute in Erlenmeyer flasks 200 c.c. each. Steam again for 50 minutes.

(b) Ten grams of basic fuchsin are shaken with 100 c.c. 96 per cent alcohol. After 20 hours this solution is filtered. (a) and (b) are mixed for use later.

3. *Loeffler's Malachite green agar.*—

Prepare one liter of broth with one-half pound of meat, as previously described, but without peptone or salt. After the sterilization, add 30 grams of agar. If the agar dissolves slowly, add 7 c.c. N-hydrochloric acid, which has to be neutralized with 7 c.c. N-potassium hydroxide solution, soon after the agar is dissolved. The agar is neutralized to litmus with sodium carbonate solution. An excess of 25 c.c. N-sodium carbonate

is added. The entire mixture is boiled again. To the hot material, 100 c.c. of a 10 per cent nutrose solution is added. (The nutrose is prepared by stirring the nutrose powder slowly into boiling water; only when this process is carried out slowly can a perfect solution of the chemical be guaranteed). The entire agar, with the nutrose, is sterilized for two hours in steam.

4. *Conradi's Brilliant Green Medium.*—

Take of Liebig's extract 20 grams (2 per cent); peptone, 10 grams (1 per cent); agar, 30 grams (3 per cent); and water to 1000 c.c. This amount of meat extract should give about the proper acidity, plus 3. If not, the reaction should be adjusted to that point. Filter through cotton, tube 150 c.c. amounts in 250 c.c. Erlenmeyer flasks and sterilize.

Then add 1 c.c. of a 1 to 1000 aqueous solution of brilliant green (Hoechst) and 1 c.c. of a 1 per cent solution of picric acid to the flasks containing 150 c.c. of melted agar. Sterilization after adding the dyes precipitates them and is unnecessary. Pour the finished medium into large Petri dishes and inoculate the surface with feces. Brilliant green does not interfere with agglutination, as does malachite green. This medium is the one of choice in isolating typhoid bacilli from feces and urine.

B. INFECTION AND IMMUNITY

First Day

1. *Action of of hemotoxic (hemolytic) serum on animal furnishing the blood cell.*

Inject two guinea-pigs intraperitoneally as follows:

No. 1. Given 3 c.c. of a strong hemolytic serum of a rabbit that has been immunized against guinea-pig red blood corpuscles: The titer of this serum should be determined with guinea-pig corpuscles (5 per cent suspension) and rabbit alexin. The serum has been heated at 56° C.

No. 2. Given 3 c.c. of normal rabbit serum, 56°, intraperitoneally.

Watch carefully for death of guinea-pig No. 1 (18 to 48 hours). Autopsy carefully; also chloroform and autopsy guinea-pig No. 2. Note conditions, particularly urine, blood in vessels, and liver.¹ Fix tissue, particularly liver, in Zenker's and alcohol, and in stained preparations examine for thrombi and necrosis.

¹ Pearce, *Journ. Med. Research*, vol. 12, 1904, p. 392.

Second and Third Day

2. Agglutination of bacteria

(a) Macroscopic method.—

Whole surface agar slants of known cultures of *B. typhosus*, *B. coli*, and *B. enteritidis* are prepared by the entire class on the second day, and on the third day each culture is suspended in 5 c.c. of normal saline containing 0.5 per cent carbolic acid. This will kill cultures in a few hours (test sterility on following day) and preserve them so that they can be used subsequently. A bouillon culture of *B. typhosus* is also planted the second day. An anti-typhoid serum, obtained by immunizing a rabbit with cultures of *B. typhosus*, heated at 56° C, and inactivated normal rabbit serum will be provided. The titer (limit of agglutinating potency) of the anti-typhoid serum will be noted. Make three series of small test-tubes containing increasing dilutions of the anti-typhoid serum in 1 c.c. saline (i.e., 1-100, 500, 1000, etc., depending on potency of serum). About 5 tubes in all are made. To each series is added, by means of a capillary pipette, from two to five drops of a suspension of one of the suspended cultures. The resulting mixture should be faintly cloudy. Control tubes should be made for each organism containing:

1. Normal saline.
2. Two tubes with the corresponding lowest dilutions (1-100; 1-500) of normal rabbit serum.

Incubate at 37° for one hour and examine and describe. Leave at room temperature and examine the next day.

(b) Microscopic method.—

Take one oese of an actively motile culture of *B. typhosus* and place it on a clean cover-slip that has been flamed. Add one drop of a 1-1000 dilution of the anti-typhoid serum; mix and invert over a hollow ground slide and seal with vaseline. Watch carefully and at frequent intervals with artificial light and high power objective, until agglutination is complete.

3. Agglutination of red blood cells (Hemagglutination)

With materials that have been prepared the class should set up the following tubes:

1. Rabbit-anti-guinea-pig serum, 56°, 0.01 c.c.+ washed guinea-pig blood, 5 per cent, 1 c.c.
2. Normal rabbit serum, 56°, 0.01 c.c.+ washed guinea-pig blood, 5 per cent, 1 c.c.
3. Rabbit-anti-guinea-pig serum, 56°, 0.01 c.c.+ washed sheep blood, 5 per cent, 1 c.c.

D. HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Aneurism is a dilatation of a vessel due to the blood pressure acting on a diseased wall.

Causes of aneurism: infection, acute or chronic—especially syphilis, various toxic substances, arteriosclerosis, traumatic.

Some forms of aneurism: saccular; fusiform; diffuse; multiple; dissecting, etc.

Relation of aneurism to the surrounding tissue; pressure phenomena.

Rupture of aneurism.

Clot formation within aneurism.

Second Day

Review of histogenesis of blood elements.

Bone marrow. Its relation to general infection with leucocytosis.

Types of leucocytosis.

Hypoleucocytosis.

Gelatinous degeneration of bone marrow.

Myelogenous leukemia.

Changes in the blood, especially the neutrophilic myelocytes. Changes in other organs.

Multiple myeloma.

Lymph nodes: Their relation to infection, and reactions of endothelium and other elements.

Anthraxosis.

Hodgkin's disease.

Lymphatic leukemia.

Changes in the lymph nodes, blood and other organs.

Status lymphaticus.

Third Day

Anemia—Primary and secondary.

Secondary anemias.

Hemorrhage.

Acute infections.

Poisons as lead, mercury, acetanilid, etc.

Blood parasites.

Intestinal parasites.

Character of blood changes.

Chlorosis.

Pernicious anemia: obscure etiology; infection suspected.

Blood changes: great reduction in number of red cells.

Poikilocytosis.

Nucleated red cells—normoblasts, microblasts and macroblasts.

Increase of color index.

Changes in other organs.

Polycythemia.

Fourth Day

Review of diseases of cardiovascular system and blood.

EIGHTH WEEK**GENERAL OUTLINE***Bacteriology*

Continuation of study of *B. coli*, *B. typhosus*, and *B. enteritidis*.

Continuation of water analysis.

Continuation of isolation of *B. typhosus* from water and stools.

Study of dysentery and paratyphoid bacilli.

Infection and Immunity

Lecture on specific precipitins; and two on pathogenesis, diagnosis, and specific therapy of infections due to the colon-typhoid group of bacteria.

Experiments on precipitins; the reaction of alexin fixation; the skin test in typhoid immunes; the action of *B. dysenteriae* (Shiga) on rabbits.

Experimental Pathology

Experimental pneumonia in rabbits. Experimental Hydrothorax; Toxic Gastritis; gastric ulcer, intersusception and volvulus.

*Histopathology and Morbid Anatomy***First Day**

Diseases of the respiratory system.

Second, Third and Fourth Days

Diseases of the gastro-intestinal tract. Typhoid fever; dysentery; cholera.

*Bacteriology***First Day**

Exercise 16. *B. typhosus* and *coli* (e).—

The cultures of the *B. typhosus* and *coli* showed no remarkable alterations, with the exception that they became somewhat larger. One-half of the eight bouillon cultures are examined for the formation of indol. Only colom bacilli produced indol.

Exercise 17. *Continuation of the identification of the organism isolated from water* (e).—

Exercise 19. *Isolation of the typhoid bacilli from stools and water (b).—*

1. (a) The extract of the horse-meat is boiled for an hour, then filtered and 20 grams peptone, 20 grams nutrose, 10 grams sodium chloride are added; the mixture is boiled again for an hour, then filtered; 60 grams of finest agar added; placed in the autoclave until melted; rendered slightly alkaline to litmus (or + 1.0) with 10 per cent sodium carbonate.

(b) 274 c.c. of Kubel-Tiemann litmus solution is boiled for ten minutes, 30 grams of lactose added and boiled for fifteen minutes.

This solution and the agar are cooled to 50° C. They are then mixed, well shaken and, if necessary, the slightly alkaline reaction (+ 1.0) restored. (Acid reaction due to splitting of the lactose). Add 4 or more c.c. of a hot sterile 10 per cent solution of sodium carbonate to obtain a final reaction of +.2 and 20 c.c. of a freshly prepared solution made by dissolving .1 gram crystal-violet B (Hoechst) in 100 c.c. hot sterile distilled water.

The finished medium is distributed in sterile flasks containing 100 c.c. each. They are sealed with tin foil, without being sterilized again. This medium gives satisfactory results only when it has not been overheated and the lactose changed.

2. (b) The fuchsin solution is filtered.

(c) Melt 100 c.c. of the agar previously prepared and sterilized. Add one gram of lactose and sterilize in Arnold for ten minutes. To 10 c.c. sterile water add one gram anhydrous sodium sulphite c.p. and 1 c.c. of a 10 per cent alcoholic solution of basic fuchsin. Sterilize for ten minutes. Add 1 c.c. of the sterile decolorized fuchsin solution to the 100 c.c. lactose agar, using sterile pipette. Pour the agar into three or four large 15 c.c. Petri dishes. Cool in air with covers off. Dry in incubator for one-half to one hour inverted. Replace covers and store in dark until used. Plates more than one day old should never be used. Plates prepared by this method should be practically colorless by transmitted light. Viewed horizontally, the agar layer should show a faint fresh color.

3. Malachite green agar: The medium is sterilized again, for two minutes, with steam. In the steam, the supernatant, clear portion of the agar is collected, with sterile precautions, in sterile flasks in the quantity of 100 c.c. each. The medium is never filtered.

Sterilize ox bile, filtered through cotton, add 1 per cent peptone, fill in test tubes and sterilize it again.

Exercise 20. *B. dysenteriae; B. paratyphosus (a).—*

Prepare some tubes of agar containing 10 per cent litmus and 2 per cent mannite: 100 c.c. of ordinary neutral agar are mixed with the litmus

solution and the mannite. Fifteen c.c are placed in test tubes and sterilized on three consecutive days in running steam for ten to fifteen minutes.

Exercise 19. *Isolation of typhoid bacilli from water (c).—*

Place in a bottle, one liter of tap water, prepare a bouillon culture of the *B. typhosus*, a 10 per cent solution of sodium carbonate, a 10 per cent solution of iron sulphate (Ferrie) and a 25 per cent solution of neutral potassium tartrate.

Exercise 18. *B. enteritidis (Gaertner) (b).—*

Mice fed with the meat poisoning organisms usually die after five to eight days. At the autopsy a liquid content is found in the small intestines; the lymph follicles are distinctly swollen; in the liver and spleen there are necrotic foci; the internal organs are removed to a petri dish and examined microscopically; numerous rods are seen which are very motile and of nearly the same size, shape and other morphological properties as the typhoid bacillus. In the liver and the spleen they are usually in pure culture; in the intestines they are frequently found mixed with other bacteria in desquamated epithelial cells. Cultures are prepared by plating the liver and intestinal contents in agar and gelatin plates; smear the contents of the intestinal tract and small pieces of the liver over lactose, litmus, nutrose agar; or, still better, Endo's fuchsin agar. A few pieces of the intestinal mucous membrane and a few necrotic foci can be fixed in formalin for sections. Prepare agar slants of the various *B. dysenteriae*, *Paratyphosus* (a) and (b).

Second Day

Exercise 16. *Typhosus and coli (f) (Repeat indol reaction).—*

Exercise 19. *Isolation of the typhoid bacilli from the stools and the water (d).—*

If there is no stool of a typhoid patient available, normal stool is mixed with a small amount of water until it has a soft consistency. Ten c.c. are mixed with one loopful of a broth culture which has previously been diluted with 2 c.c. of water. Litmus, nutrose, lactose agar—or fuchsin agar—plates are poured. (About a layer of 2 mm. in height). Let the plates stand open and inverted for one-half hour in the incubator to evaporate the condensed water. By means of the Dalli, a small amount of stool is smeared on the surface of the first, then on the second and then on the third plate, in the manner described above. The plates are placed in the incubator in an inverted position.

3. In a similar manner, the malachite green agar can be inoculated; the medium is prepared as follows:

To 100 c.c. of the medium prepared yesterday, add 3 c.c. of bile and 1.9 c.c. of 0.2 per cent aqueous solution of malachite green (Malachite green cryst, chemically pure (Hoechst). The mixture is carefully shaken and poured into plates.

The demonstration of the Typhoid Bacilli in water is done by means of precipitation; in the bottle containing one liter of tap water, and which has been kept at room temperature for forty-eight hours, one loopful of typhoid bacilli is inoculated. Shake the bottle carefully and pour the contents in a high stoppered measure-glass (avoid infection), add 4 c.c. of a 10 per cent sodium carbonate solution and 3.5 c.c. of a 10 per cent ferric sulphate solution; stir with a glass-rod and let the precipitate settle by placing the cylinder in an ice-chest for two hours: remove carefully the supernatant, clear fluid; place the precipitate in sterile test-tubes and add one-half of the volume of a 25 per cent solution potassium tartrate; shake again until the major part is dissolved. To 1 part of the solution, add 2 parts of sterile broth and smear 1 c.c. each, on four litmus, nutrose, lactose agar.

When the water is very hard, an addition of 3.3 c.c. of iron oxy-chloride solution will also cause a precipitate; let it settle, filter through sterile filter paper and smear the retained precipitate on three large plates of fuchsin agar, or litmus, nutrose, lactose agar, respectively, and a few gelatin plates.

The filtrate, filter, funnel, and bottles, are disinfected.

Exercise 18. *B. enteritidis* (Gaertner) (c).—

On the gelatin plates, prepared from the organs, numerous colonies, have grown. They cannot be distinguished from those of the *B. coli*. On the lactose, litmus agar, the blue colonies which have grown, are distinguished from the typhoid colonies by their size and their opacity. On the gelatin plates, prepared with material from the intestinal tract, the colonies of the *B. Enteritidis* cannot be distinguished from the colonies of the *B. coli*. On the litmus, nutrose, lactose agar plates, they appear blue, whereas the *coli* are naturally red. The microscopic examination shows actively motile rods of similar appearance to the typhoid bacilli. From a few colonies, the following cultures are prepared:

Gelatin stab; agar stab; bouillon; milk; glucose agar, with neutral red.

Exercise 20. *B. dysenteriae*; *B. paratyphosus* (b).—

The examination of the dysentery and paratyphoid cultures shows rods of the same size and shape as the typhoid bacilli. The dysentery bacilli are non-motile, the paratyphoid bacilli are very motile. From the various strains, *B. dysenteriae* (type Shiga-Kruse, type Y, type Flexner,

Manila, type Harris-Gray, type Shiga-Tsuchiya). Paratyphosus (A); Paratyphosus (B); the following subcultures are prepared:

Gelatin plates, agar plates, gelatin stab, agar stab, shake cultures in glucose agar, with and without neutral red, bouillon, potato, milk, litmus whey. Smear some litmus nutrose lactose agar plates with some of the organisms. Liquefy several tubes of litmus mannite agar, and inoculate them with the various dysentery bacilli, paratyphoid, typhoid and coli bacilli, respectively. If time permits, inoculate the different types of dysentery bacilli, paratyphoid, coli and typhoid bacilli into Hiss glucose, maltose, saccharose, lactose, mannite, or dextrin-serum-water-litmus-media. (One part of ox serum, three parts of distilled water with 1 per cent litmus and 1 per cent carbohydrate; sterilized on three consecutive days in a steam sterilizer.)

Third Day

Exercise 19. *Isolation of the typhoid bacilli from the stools and the water (e).—*

On the litmus nutrose lactose agar plates, numerous colonies have developed. They can very readily be distinguished. The Colon Bacilli colonies are red; the typhoid bacilli alone have not fermented the medium which remains alkaline and, therefore, blue. The streptococci have been inhibited in their growth by the crystal violet. From all of the other blue colonies, the typhoid organisms are distinguishable as dew-drop-like, transparent colonies. They have never a double margin or bluish tinge. The size varies between one to three m.m.

They are identified by means of the microscopic agglutination test. Several colonies are transplanted on slanted agar.

In a similar manner, the plates prepared with the water are examined. Considerable difficulty is offered, on account of the fact that numerous water bacteria resemble, in their growth, the typhoid colonies. It is best to wait a few days, keeping the agar plates at room temperature; the water bacteria colonies turn brown or greenish, whereas the typhoid colonies remain blue.

From the gelatin plate colonies, agar slants are prepared.

On the fuchsin agar, the results and the growth are analogous. The coli colonies are intensely red, shiny, raised, of a greenish tinge, resembling the dried fuchsin. The diameter is usually more than $\frac{1}{2}$ c.c. The typhoid colonies are colorless or pinkish, shiny, and not more than 2 mm. Other bacteria grow very poorly.

On the malachite green agar, if the medium has been prepared properly, the growth of the colon bacilli is inhibited, or they appear as whitish formations, microscopically, a brownish color. The typhoid colonies are fine, transparent and, under the microscope, colorless, granulated, with a lobulated margin.

Exercise 20. *B. dysenteriae*; *B. paratyphosus* (c).—

The biological properties of *B. dysenteriae* are practically the same as those of the typhoid bacillus. The appearance of the gelatin, agar, bouillon, and potato cultures is identical. Glucose is not fermented. Milk is not coagulated. Neutral red is not reduced. Litmus whey is slightly reddened but not turbid. On the litmus nutrose, lactose agar, colonies are small, blue, but less transparent than those of the typhoid. The dysentery bacilli of the Shiga type do not ferment the mannite agar or decolorize only the deeper layers. All the other bacteria reduce and change the medium from blue to red.

The paratyphoid bacillus "A" is absolutely identical with the typhoid bacillus, with the exception that it ferments glucose, and neutral red. The paratyphosus "B," is, in cultural respects, more closely allied to the *B. coli*: the growth on gelatin, agar and potato is more abundant. The litmus whey, light reddish and turbid; glucose, fermented; neutral red, reduced; milk, not coagulated; colonies on the litmus, nutrose, lactose, agar: blue, but larger and opaque.

A definite classification can only be made by means of the Agglutination test.

The cultures are preserved for further observation.

Fourth Day

Exercise 19. *Isolation of typhoid bacilli from the stools and the water* (f).—

The colonies transplanted on slanted agar are studied in the manner discussed in the various exercises by means of the identity reactions.

Exercise 18. *B. enteritidis* (Gaertner) (d).—

Indol reaction with the bouillon culture is occasionally positive, but more frequently negative. The milk culture is unchanged.

Exercise 20. *B. dysenteriae*; *B. Paratyphosus* (d).—

The cultures have developed further. The surface cultures of the Dysentery bacilli show fine lines and clefts, in the same manner as the typhoid colonies. The Paratyphosus A, and particularly the type B, does not show these changes. (Prepare a culture of the *B. mallei* on slanted glycerin agar).

INFECTION AND IMMUNITY

First and Second Days

Phenomenon of precipitation.—A rabbit anti-human serum, obtained by immunizing rabbits with human blood serum, will be furnished; also the serum of a normal rabbit. The titer of the serum will also be given, that is to say, the smallest amount of human serum which with a fixed dose of the anti-human serum (.3 c.c.) will give a clear cut reaction. The amounts of human serum in the following protocol should be modified in accordance with the titer of the serum, so as to reach the maximum.

Tube 1.	Human serum, .01	c.c. + rabbit anti-human serum, 56°, 3 c.c.
Tube 2.	Human serum, .001	c.c. + rabbit anti-human serum, 56°, 3 c.c.
Tube 3.	Human serum, .0001	c.c. + rabbit anti-human serum, 56°, 3 c.c.
Tube 4.	Human serum, .01	c.c. + normal rabbit serum, 56°, 3 c.c.
Tube 5.	G. pig serum, .01	c.c. + rabbit anti-human serum, 3 c.c.
Tube 6.	Monkey serum, .01	c.c. + rabbit anti-human serum, 3 c.c.
Tube 7.	Monkey serum, .01	c.c. + normal rabbit serum, 3 c.c.

The tubes should be watched, at room temperature, for an hour or two for the presence of cloudiness and then left over night. The following morning the appearance of sediment should be noted. (This test may be carried out with horse-serum and a rabbit anti-horse-serum, by others of the class).

2. *Production of precipitins.*—Two or more members of the class will immunize two rabbits against horse serum by the intensive, intravenous method as outlined for the production of bacteriolysins.

3. *Production of Agglutinins.*—Groups of students will produce agglutinins to the typhoid bacillus.

(a) By the rapid intravenous method of injection similar to that outlined last week for bacteriolysins.

(b) By the slower, subcutaneous method with seven day intervals, using in both (a) and (b) four doses of one-fourth of a killed culture of *B. typhosus* (56° for one-half hour, or simply the addition of .5 per cent carbolic acid) on each injection.

4. *Reaction of alexin fixation (Bordet-Gengou Phenomenon).*—This extremely important reaction is a test for either antigen or antibody when one of the factors is known to be present. The essential tubes necessary to demonstrate this phenomenon may be illustrated by the following tubes for which the materials will be furnished. (The doses given are not fixed amounts, but depend on the potency of the material used, and changes may be made when the experiment is actually given out).

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Tube 1.	Typhoid vaccine, 0.1 c.c.+ anti-typhoid serum, 56°, .2 c.c.+ alexin guinea-pig, 0.1 c.c.
Tube 2.	Typhoid vaccine, .02 c.c.+ anti-typhoid serum, 56°, .2 c.c.+ alexin guinea-pig, 0.1 c.c.
Tube 3.	Typhoid vaccine, .01 c.c.+ anti-typhoid serum, 56°, .2 c.c.+ alexin guinea-pig, .1 c.c.
Tube 4.	Typhoid vaccine, .1 c.c.+ normal rabbit serum, 2 c.c.+ alexin guinea-pig, .1 c.c.
Tube 5.	Typhoid vaccine, .02 c.c.+ normal rabbit serum, 56°, .2 c.c.+ alexin guinea-pig, .1 c.c.
Tube 6.	Cholera vaccine, 0.1 c.c.+ rabbit anti-typhoid serum, 56°, 2 c.c.+ alexin guinea-pig, .1 c.c.
Tube 7.	NaCl 0.1 c.c. 0.1 c.c.+ anti-typhoid serum, 56°, 0.2 c.c.+ alexin guinea-pig, .1 c.c.

Allow these tubes to stand for one hour in the thermostat and then add to each tube 1 c.c. 5 per cent suspension of sheep corpuscles that have been treated with four M.H.D. (minimal hemolytic dosage) of a rabbit anti-sheep serum. Watch hemolysis in the tubes for two hours, noting time when it is complete. Allow tubes to stand until the following day, and observe them again.

Second and Third Days

5. *A skin test in typhoid immunes (a presumptive evidence of protection.)*

Certain students, who volunteer, will be divided into two lots of

(a) Those who have recovered from typhoid fever, or those who have been vaccinated against typhoid fever, and

(b) Those who have not been vaccinated or recovered from typhoid fever (controls).

These two series will try the skin test on one another, which is done by abrading with a von Pirquet chisel two small spots about 2 mm. wide on the forearm or upper arm in such a manner as to remove the epidermis without drawing blood. Into one of these spots is rubbed, with a sterile toothpick, a "typhoidin" solution by growing *B. typhosus* in 5 per cent glycerine bouillon and evaporating to 1/10 its volume after five days. Into the other spot will be rubbed a corresponding solution of 5 per cent glycerin bouillon in which no typhoid bacilli have been grown. The induration and areola surrounding these abrasions should be noted and measured at six and twenty-four hours.

6. Action of Shiga dysentery toxin on rabbits.—

Two normal rabbits are given injections of *B. dysenteriae*, Shiga type, in the following manner:

No. 1. $\frac{1}{4}$ of a 24-hour slant agar culture, subcutaneously.

No. 2. $\frac{1}{10}$ of a 24-hour slant agar culture, intravenously.

These animals are autopsied when they die, and the lesions, particularly those of the intestine, noted and tissues preserved in the usual manner for histological examination.

EXPERIMENTAL PATHOLOGY

1. *Production of pneumonia rabbits.*—Etherize rabbit. Introduce soft rubber tube into trachea, passing it well down. Inject 5 c.c. of suspension of pneumococci followed by 5 c.c. air to blow material from the tube. Follow temperature, leucocytes, and chloride out-put in urine.

2. *Hydrothorax.*—Connect dog with kymograph to register blood pressure and respiration. Produce hydrothorax by injection of salt solution gradually increasing the amount. Record results as shown by kymographic tracings. Examine the atelectatic lung.

(Emphysema and asthmatic condition are illustrated by anaphylactic reaction in guinea pigs.) (See 12th week).

3. *Action of corrosive poisons.*—Deeply anaesthetise rabbits—introduce stomach tube and pass through it in different amounts, strong solution of potassium hydroxide or concentrated sulphuric acid, carbolie acid, and corrosive sublimate. Chloroform the animals. Make an autopsy after fifteen minutes.

4. *Gastric ulcers.*—Produce in dog by cutting out a bit of mucosa from stomach wall. When stomach is open, also inject agar-agar in mucosa. Examine forty-eight hours later.

5. *Intussusception and volvulus.*—Produce in dog. Put in a few retaining stitches to insure against spontaneous righting of the intestine. Examine after forty-eight hours.

MORBID ANATOMY AND HISTOPATHOLOGY

First Day

Respiratory system.—Congenital anomalies.

Deformities of the chest.—Congenital and due to disease.

Rhinitis.—Acute, chronic, atrophic and hypertrophic.

Pharyngitis, acute and chronic.

Lymphatic system of the nasopharynx; its relation to infection.

Second Day

Stomatitis: Aphthous—Herpes labialis.

Koplik's sign—strawberry tongue.

Thrush—*oidium albicans*.

Noma.

Parotitis—Specific and non-specific. Relation of latter to inter-abdominal infections.

Oesophagus—post mortem rupture of; **Strictures**—dilatation—diverticuli.

Hemorrhage—Perforation.

Stomach—Post mortem changes and rupture of.

Hour glass, dilatation, pyloric stenosis, gastropptosis.

Gastritis—Acute. Membranous—Phlegmonous—Chronic atrophy of mucous membrane.

Action of corrosive poisons on.

Peptic ulcer—hemorrhage, perforation following; relation of to carcinoma.

Varices and hemorrhage.

Foreign bodies and parasites of.

New growths of stomach and oesophagus.

Intestines—congenital anomalies.

Meckel's diverticulum.

Congenital herniae.

Diverticuli.

Megacolon.

Dilatations—acute and chronic.

Stenosis.

Volvulus.

Intersusception—Agonal.

Herniae: Strangulation; Incarceration.

Intestinal obstruction—Relation of chronic adhesions to.

Hyperemia—active and passive.

Varices.

Edema.

Hemorrhages.

Thrombosis and embolism.

Exercise.—Examination of sections from autopsy material.

Third Day

Inflammation of the intestines; Catarrhal—acute, chronic; Enteritis and colitis.

Specific inflammations.

Typhoid fever—mode of infection—Bacteremia—Rose spots.

Intestinal lesions; location—ileum, especially in region of Peyer's patches; appendix, occasional lesions in large intestines, rarely ulcers in stomach.

1. Stage of lymphatic enlargement.

Endothelial hyperplasia.

2. Stage of necrosis.

3. Stage of ulceration.

4. Stage of repair.

Alterations in other organs, especially liver and spleen and mesenteric lymph nodes and lungs.

Complications—Hemorrhages, early—late; perforation—peritonitis.

Fourth Day

Relapses in typhoid fever.

Complications and sequelae of typhoid fever—Secondary invaders.

Bone lesions.

Lesions in cardiovascular apparatus—Thrombosis.

Gall, bladder and liver; kidneys.

Lesions in central nervous system—acute alteration, meningitis.

Peripheral neuritis.

Appendicitis—acute.

Peritonitis following; Adhesions; Abscess formation; Rupture of appendix abscess; Liver abscess following appendicitis.

Bacillary dysentery.

Intestinal lesions.

Asiatic cholera.

Intestinal lesions.

Diphtheritic colitis.

NINTH WEEK

GENERAL OUTLINE

Bacteriology.—

Continued study of *B. enteritidis*; *B. dysenteriae*.

Study of *B. tuberculosis* in sputum and cultures.

Study of *B. mallei*; *B. abortus bovis*.

Phosphorescent bacteria; *B. leprae*.

B. pseudo tuberculosis ovis.

Methods of milk analysis.

Infection and Immunity.—

Two lectures on tuberculosis.

Study of the conglutination reaction and the reaction of animals to tuberculin.

The V. Pirquet test for tuberculosis.

Experimental Pathology.—

No assigned work.

Histopathology and Morbid Anatomy.—

First Day

Amoebic dysentery; Diseases due to intestinal parasites.

Second, Third and Fourth Days

Tuberculosis.

BACTERIOLOGY

Exercise 21. *Tuberculosis* (a).—

The sputum to be examined for tubercle bacilli is poured into a Petri dish and, for examination, the yellowish pus-like clumps are selected. One of these clumps is squeezed between two slides and then a cover-slip is smeared with the material. The staining is done as follows:

1. Fix the preparation by passing it three times through the flame.
2. Cover the preparation with undiluted carbol fuchsin, boil once, and let it stand for two minutes.
3. Rinse in water.
4. Decolorize by placing the preparation in 3 per cent hydrochloric acid alcohol until it has only a faint pinkish tinge. (One-fourth to one-half minute).
5. Rinse thoroughly with water.
6. Stain very quickly (about 10 seconds), with diluted Loeffler's methylene blue; or with Bismark-brown.

The tubercle bacilli appear as red, slender, slightly curved or bent rods. The other bacteria, diplococci or streptococci or tissue particles, are stained blue or brown.

Frequently the material contains only a very few bacilli which, by the ordinary method of staining, cannot be demonstrated. Therefore, the sputum must be concentrated by using (1) hydrogen peroxide, or (2) antiformin with or without ligroin.

(1) Place a large amount of sputum in a sedimentation glass. Add a small amount of hydrogen peroxide and stir the mixture with a glass rod. The gas produced breaks up the larger particles, and the mixture separates in three layers: a supernatant frothy layer, a watery layer, and a lower part which consists of small clumps. In the sediment an enrichment of the tubercle bacilli has taken place and the organisms are found more readily.

(1) Ligroin method. Five c.c. are mixed with 20 c.c. of a 20 per cent solution of antiformin in a stoppered graduate, and shaken. At room temperature, or better still at incubator temperature, the mixture will become homogeneous. The process can occasionally be accelerated by adding about 20 c.cm. of water. In about one or two hours, the other organisms have been destroyed and the material can be used for inoculation. For microscopic examination, a small amount of ligroin is added (a layer of 3 to 5 mm.) and shaken properly to produce a heavy emulsion. Let stand until a distinct separation has taken place. This separation takes twenty of thirty minutes; by placing the glass in a water bath at 60° C, quicker results are occasionally obtained. Withdraw several loopfuls of material from the layer which is just below the ligroin and prepare coverslips as above explained. Frequently, guinea pigs must be inoculated for diagnosis. (See under Infection and Immunity).

The cultivation of the tubercle bacillus directly from the sputum is relatively difficult, but should be carried out by every student, if possible. A sputum should be selected which is poor in extraneous bacteria, because these contaminants overgrow the developing tubercle bacilli. The culture medium to be used is the following:

Shredded agar	10	gr.
Sodium chloride	5	gr.
Sodium carbonate cryst	1.43	gr.
Distilled water	1000	gr.

The medium is poured into Petri dishes.

On this culture media the tubercle bacilli develop, owing to the mucus; all the other bacteria are relatively inhibited in their growth. The sputum is washed in sterile saline and then several plates are smeared with the material. The plates are kept in moist chambers at a temperature of 37 degrees.

Exercise 20. *B. Dysenteriae*; *B. Paratyphosus* (e).—

The bouillon cultures of dysentery and paratyphosus are examined for indol. With the exception of the milk and litmus whey cultures, the tubes can be destroyed.

Exercise 22. *Glanders* (a).—

As the work with glanders bacilli is associated with considerable danger, it is carried out by only a few students and with great precaution. Of the glanders bacilli culture prepared yesterday, two loopfuls are suspended in 2 c.c. of saline solution and a male guinea pig is inoculated intraperitoneally with 1 c.c. of this suspension. To avoid the formation of ulcers in the skin, the needle is cleaned with cotton before the injection is made. The seat of injection is covered with collodium, and special care taken that the bedding in the cage is not contaminated. Examine the guinea pig daily. (Note swelling of testicles, periorechitis).

Second Day**Exercise 21.** *Tuberculosis* (b).—

To obtain a general knowledge of the morbid anatomy of a guinea pig, infected with tuberculosis, the animal which has been infected three weeks ago is weighed and the loss in weight is recorded. It has been killed either with tuberculin or with chloroform. The post mortem is conducted very carefully, with sterile instruments, as an attempt should be made to isolate the tubercle bacilli from some of the lesions. At the seat of the inoculation, usually when the material has been introduced under the skin, an ulcer has developed. The inguinal lymph nodes are enlarged and can be felt distinctly from outside by palpating. When opening the ulcer, one finds cheesy material; the inguinal lymph nodes show numerous cheesy nodules. If there has been no material inoculated under the skin, these lesions are absent. Of the organs in the abdominal cavity, the liver and spleen are particularly enlarged and studded with tubercles; the large foci resemble anemic infarcts. The omentum is curled up and converted into a cheesy mass, which is dotted outside with nodules; the peritonem and the following lymph nodes are particularly diseased; crural, iliac, paraliae, paraortic, retroperitoneal, *bronchial*, submaxillary, etc. In the lungs numerous tubercles are also seen.

The organs are removed into sterile Petri dishes and examined primarily by smears and sections. The smears are stained according to Ziehl's method as described above. Between the blue cells, numerous tubercle bacilli are seen.

Some of the organs can be fixed in bichloride solution and used for sections.

To obtain cultures, a small focus of the spleen is cut, by means of sterile scissors, into numerous small pieces. Several of these particles are rubbed, by means of the platinum wire, on the surface of slanted serum or glycerin brain agar tubes. In this manner, about six tubes each are inoculated, sealed with paraffin and incubated.

Beef serum without or with an addition of 2 per cent to 2.5 per cent glycerin solidified at a temperature of 67 degrees, is used. The brain agar is prepared as follows: Finely chopped brain is mixed with an equal amount of water, and by constant stirring, heated and boiled for fifteen minutes, then pressed through cheesecloth. The filtrate should be jam-like. Without neutralization this filtrate is mixed with an equal amount of a 2 per cent aqueous solution of agar; add 3 per cent glycerin, and sterilize. The tubes should be carefully mixed and coagulated rapidly, before the brain and agar layer can separate.

Several organ pieces are smeared on the surface of glycerin agar plates. To avoid drying, they are placed inverted into a large, moist, chamber dish in which, at the bottom, a cotton plug, soaked with bichloride, is placed. Incubate. Observe daily whether there is still sufficient fluid in the chamber.

Examine specimens of tuberculous material derived from animals, particularly bovine and avian material.¹ Note that the bovine tubercle bacilli are usually somewhat thicker and shorter than the human bacilli. In the tuberculous lesions of birds, the tubercle bacilli are very numerous and are seen as very slender rods or clumps and clusters.

Examine samples of urine, or pleural exudate from human beings, for tubercle bacilli. These fluids are usually very poor in tubercle bacilli. They must be centrifugalized, and the sediment examined. If the tubercle bacilli are not found, use the antiformin method.

The microscopical diagnosis of tuberculosis is frequently unreliable, as numerous other bacteria are also acid fast. For example: the *Smegma bacillus*, an ordinary saprophyte is found in the urine when the sample is improperly collected. Or, in guinea pigs, the pseudo-tubercle bacilli, which are also acid fast, produce tuberculous-like lesions. The student is advised to stain several preparations, made from cultures of these organisms, according to various staining methods (the stain for tubercle bacilli, Gram. thionin). The *Smegma* bacilli are acid fast only; they become decolorized with absolute alcohol. The pseudo tubercle bacilli show microscopically no difference, but grow very readily on the ordinary culture media.

¹ Stain some preparations in which the tubercle bacilli are very few—by Much's method. (See page 276, Muir & Ritchie).

To differentiate the Smegma bacilli from Tubercle bacilli, carry out the following test: Smear, on different parts of one slide, some material from a culture of tubercle bacilli and of smegma bacilli; handle and treat as customary for the tubercle bacillus, but decolorize by immersing the slide for a few seconds in a 30 per cent aqueous solution of concentrated nitric acid. Decolorize, in 95 per cent alcohol, for at least two minutes. Wash in water. Stain in Loeffler's methylene blue solution for one minute. Wash in water. Dry and mount.

Examine several cultures of the various tubercle bacilli—strains grown on glycerin broth; note particularly branched organisms. Stain some preparations also according to Gram's method.

Exercise 23. *B. Abortus Bovis* (a).—

Study the anatomical lesions of a guinea pig, which has been inoculated three months previously with *B. Abortus Bovis*. Small, Gram positive rods are occasionally seen microscopically. It is necessary to prepare the preparations from the enlarged spleen. Inoculate, with liver and spleen material, a few tubes of slanted glycerin-agar (plus 1.5). Seal the tubes with paraffin and incubate at 37 degrees.

Exercise 24. *Milk Analysis* (a).—

The work done in this exercise is conducted according to the standard methods for the bacteriological examination of milk. A.P.H. 1910, and the Commission of Milk Standards, Reprint 141, Public Health Reports, August 22, 1913.

Sterilize at least ten straight 1 c.c. pipettes, carefully wrapped in paper, for one hour at 160° C. Have on hand 20 tubes of agar (reaction plus 1 per cent), sterile Petri dishes; 5 short, wide-mouthed, eight-ounce bottles containing 99 c.c., and tubes containing 9 c.c. sterile, distilled water. The bottles and tubes should be plugged with long fibre, non-absorbant cotton.

Exercise 26. *Light Producing Bacteria* (a).—

For the cultivation of photogenetic bacteria, a salt water fish is kept at room temperature until phosphorescence is noted. Prepare agar with 3 per cent sodium chloride. Pour plates and smear on the surface a small amount of the material where the decaying fish is most prominently phosphorescent. Incubate the plates at room temperature.

Third Day

Exercise 25. *Leprosy (Paratuberculosis)* (a).—

Prepare smears or examine section of leprous tissues, which have been stained, by means of the tubercle bacilli method. Also, with ordinary alkaline dyes and by Gram's method. Note the enormous numbers within the protoplasm of the round, mononuclear cells. Examine also the lymphatics carefully.

If fresh material of rat leprosy is available, make smear preparations and stain as mentioned above. If possible, compare the staining reactions by adding, to the smear, some tubercle bacilli (the bacillus of leprosy is less resistant to decolorization).

Study by means of stained preparations, the supposed cultivated leprosy bacilli from pure cultures. Note particularly the pigment production of Clegg's & Duval's Bacillus. Study also Kedrowski's Bacillus and William's Streptothrix.

Exercise 27. *Pseudotuberculosis* (a).—

In the organs of guinea pigs or rabbits, the formation of nodules is occasionally to be seen, which resemble, anatomically, the true tubercle. These nodules are of diagnostic importance, because they are not due to the tubercle bacillus, but to the pathogenic action of other bacteria; a special study should be made of one of these organisms. The condition in the inoculated animals is, for the reasons explained above, usually called pseudo-tuberculosis.

A guinea pig, previously inoculated with the bacillus pseudo-tuberculosis ovis (caseous lymphadenitis of sheep), is autopsied; the organs are examined, as usual; a slender, or rather plump, motile, Gram positive bacillus is seen. Cultures are prepared on agar, gelatin and serum.

Pieces of the tissues are fixed in concentrated bichloride and prepared for sections. Note the marked caseation, the absence of giant cells, and the scarcity of large, epithelioid cells.

Exercise 23. *B. Abortus Bovis* (b).—

On the agar cultures, fine, punctiform, gray-whitish colonies, with smooth edges; in transparent light; bluish; have developed. Stain preparations by means of Giemsa method, with ordinary aniline dyes and according to Gram. The bacilli appear as short, thick rods, which resemble, on account of the staining reactions, Diphtheria bacilli. Return the cultures to the incubator for further observation.

Exercise 26. *Light Producing Bacteria* (b).—

If a pure culture has been obtained of photogenetic bacteria, inoculate 3 per cent sodium chloride broth and agar.

The phosphorescence is very plain when shaking the broth.

Exercise 25. *Paratuberculosis* (b).—

Paratuberculous Enteritis, being histologically similar to Leprosy, gives very instructive preparations when stained by means of the tubercle bacilli method. Examine sections and cultures of the Bacillus paratuberculosis.

In this exercise, it is particularly important that the student stain as many preparations as possible, by means of various aniline dyes, and note the differences of staining reactions.

Exercise 27. *Pseudotuberculosis* (b).—

Only the cultures incubated at 37 degrees show a poor growth. On agar, they are remarkably dry, sealy; on the serum, distinctly yellowish.

The cultures resemble somewhat the diphtheria bacillus. Prepare a pure slant and deep culture for a comparative study in connection with diphtheria.

Various other organisms, for example, streptothrices, bacillus pseudotuberculosis rodentium (see plague), etc., cause the formation of nodules in smaller animals.

Exercise 24. *Milk Analysis* (b).—

(1) The certified milk is plated in a dilution of 1:10, 1:100, 1:1000, 1:10,000. Melt 18 agar tubes, keep in a water bath at a temperature from 40 to 45° C for at least fifteen minutes.

Shake the certified milk sample twenty-five times, then with a sterile pipette transfer 1 c.c. to 9 c.c. sterile dilution water, rinsing the pipette by drawing dilution water to the mark and expelling; this gives a dilution of 1:10. Transfer, with a fresh, sterile pipette, 1 c.c. of milk sample to 99 c.c. dilution water, rinsing the pipette as before. This gives the dilution 1:100.

Shake the dilution 1:100, 25 times; then with fresh, sterile pipette transfer 1 c.c. to 9 c.c. and 99 c.c. dilution water, rinsing the pipette to the mark as before. This gives the dilutions 1:1000 and 1:10,000.

Shake the dilutions 1:10 twenty-five times, then with a sterile pipette transfer 1 c.c. to a Petri dish, using care in raising the cover only as far as necessary to insert the end of the pipette. Pour the contents of an agar tube into the plate. Carefully and thoroughly mix the agar and diluted milk in the Petri dish, by rotary motion. Allow the agar to harden for at least fifteen minutes at room temperature; place the dish, bottom down, in the incubator. Repeat the same process with the dilution 1:100, 1:1000, and 1:10,000. Control plates should always be made with each series of milk plates, for the water, agar, air, Petri dishes and pipettes.

(2) The skimmed market milk is plated, in duplicate, in the dilution 1:100; 1:10,000, 1:100,000, and 1:1,000,000. The dilutions are prepared as explained above. The dilutions are transferred in 1 c.c., each in two Petri dishes, and mixed with agar, as already explained. The controls are the same as for (1).

All of the plates are incubated for forty-eight hours at 37° C.

(3) The examination of the milk sediments, particularly the estimation of leucocytes, was formerly considered to be of value for detecting inflammatory conditions of the udder. Milk showing a high leucocytic content is unfit for human consumption. Mixed milk containing more than 500,000 leucocytes to the c.c. should be excluded from the market. However, the commission of milk standards concludes that the ordinary bacteriological analysis is quite sufficient for the daily routine.

The following exercise illustrates one of the methods ordinarily used in dairy bacteriology: 10 c.c. of the individual milk sample is placed in a sedimentation tube, heated to 70 degrees for 10 minutes, then shaken and subsequently centrifugalized twenty minutes at 1,200 revolutions per minute. After centrifugalizing, the cream and the supernatant milk are removed, with the exception of the last $\frac{1}{2}$ c.c., by aspirating with a pipette and wiping the walls of the tube with a cotton swab. After mixing the sediment with a glass-rod, enough of the emulsion is placed in an ordinary blood counter to fill the cell exactly. The preparation is allowed to stand for a minute or two. The count is made with one-sixth objective and one-inch eye piece. The average number of cells per smallest square (count from 100 to 400 squares, according to the number of cellular elements obtained) when multiplied by 200,000 gives the number of cells per cubic centimeter in the original milk. With the rest of the sediment make smears, fix in ether-alcohol, and stain by the Giemsa method. Examine particularly for streptococci. A milk showing both an abundance of long chain streptococci and pus should be condemned as unsafe.

Exercise 26. *Light producing bacteria (c).—*

Examine the cultures in a dark room. Make subcultures, if possible. Occasionally it is advisable to smear a few more plates from the phosphorescent areas. *Prepare*, for the Exercise on *Fungi*, six bread- flasks.

Cut the bread into slices and heat it until overcasted (hot air over). Crush it fine. The dry, powdered bread is filled to a depth of about one-fourth of an inch in small 30-50 c.c. Ehrlenmeyer flasks previously cleaned, plugged and sterilized. Water is added, to render the mass thoroughly moist. The bread flasks are steamed for one-half hour on each of three successive days.

Fourth Day

Exercise 21. *Tuberculosis (c).—*

If on the various cultures an abundant growth is noticed, they should be examined, and, if due to contamination, as is most frequently the case, they are eliminated. Examine milk for tubercle bacilli. The milk is

centrifugalized. The tubercle bacilli are found in the sediment as well as in the cream. The coverslip preparations made from the cream, are best fixed in ether-alcohol, equal parts, and afterwards rinsed very quickly in chloroform. The antiformin method can also be used and is particularly successful when the material is to be inoculated.

Use the following method:

Milk	5 c.c.	25% Anti-formin	10 c.c.
Ether	5 c.c.	Saline solution	25 c.c.
Absol. Alcohol	5 c.c.		

Mix carefully and centrifuge.

Exercise 24. *Milk Analysis (c).*—

Count the number of colonies on the plates. The whole number should be determined; the results are expressed in accordance with the rules of the Standard Methods. Certified milk usually shows a count of 4000 to 8000 bacteria per c.c. A count of 100,000 bacteria to a cubic centimeter is considered a serious contamination, and is found in the milk samples given for these exercises.

From the plates showing numerous pin-point colonies, transfer 10 or more to broth; grow fifteen to twenty-four hours and examine for streptococci. The presence of these organisms is considered sufficient evidence to exclude the milk until after satisfactory examination of the cows.

INFECTION AND IMMUNITY

First Day

1. *Conglutination reaction.*—

With material that will be supplied, the following experiment is carried out:

- Tube 1. Washed guinea pig blood 5%, 1 c.c.+ bovine serum 56°, 0.2 c.c.+ fresh horse serum, 0.2 c.c.
- Tube 2. Washed guinea pig blood, 5% 1 c.c.+ bovine serum, 56° 0.2 c.c.+ NaCl 0.2 c.c.
- Tube 3. Washed guinea pig blood, 5% 1 c.c.+ NaCl 0.2 c.c.+ fresh horse serum, 0.2 c.c.

Watch the tubes carefully, at intervals for an hour, at room temperature, agitating them from time to time. Note the difference in appearance between hemagglutination and cong lutination.

First and Second Days**2. The effect of tuberculin on tuberculous animals.**

Three weeks previously, two members of the class will have given normal guinea pigs intraperitoneal injections of human sputum that has, on specific staining, been shown to contain tubercle bacilli. These animals are prepared in the following manner:

- (a) Two weighed guinea pigs (Nos. 1 and 2) are given intraperitoneal injections of 1 c.c. each of tuberculous sputum that has been diluted 1 to 4 with sterile saline solution. One or both of these pigs may die from contaminating organisms (Note in counter-stained preparation).
- (b) Three weighed guinea pigs (Nos. 3, 4 and 5), are given injections of tuberculous sputum that has been treated by "antiformin." Antiformin acts on organic material by liberating chlorin which kills the ordinary bacteria in sputum, but has relatively less effect on tubercle bacilli, owing to their waxy envelope. Add to 2 c.c. of tuberculous sputum a small amount of sterile saline; shake, and add an equal volume of 15 per cent antiformin. Mix and allow to stand for thirty minutes. Centrifugalize. Remove supernatant fluid. Stain smear of sediment for tubercle bacilli and make cultures of it for control. Wash in saline (10 c.c.), remove excess, and add 4 c.c. of sterile saline; shake, and inject 1 c.c. intraperitoneally in each of the three guinea pigs.

Note carefully the condition of these injected guinea pigs, temperature, weight, size of inguinal lymph nodes, etc., during the three weeks period. If any of the animals die, make careful autopsy, cultures, and save tissue for histological examination.

On the first day of this week, the three animals of series "B" are treated as follows:

- G. P. No. 3. Left as control.
- G. P. No. 4. Given 0.5 c.c. Koch's old tuberculin (OT) subcutaneously.
- G. P. No. 5. Given 0.5 c.c. 5 per cent glycerin bouillon concentrated to 1/10 volume.
- G. P. No. 6. Normal G. P. given 0.5 c.c. Koch's old tuberculin subcutaneously.

In twelve hours No. 4 will be dead. Autopsy carefully, noting lesions with great care and preparing them with lesions in No. 3 and No. 5 which have been chloroformed. Save tissues from No. 4 and one of the others. Make cultures for growth of tubercle bacilli (see under bacteriology). Chloroform also guinea pigs Nos. 1 and 2.

3. The V. Pirquet test for tuberculosis:

If an individual with tuberculosis is available, a skin test similar to the one noted under typhoid will be made on the patient's skin with OT (Koch), controlled by a spot of glycerin bouillon. Further controls are made on normal individuals.

HISTOPATHOLOGY AND MORBID ANATOMY**First Day**

Amoebic dysentery.

Intestinal lesions. Liver abscess; pulmonary lesions.

Balantidium coli.

Intestinal parasites.

Trichina.

Tenia solium, saginata, and Echinococcus.

Hydatids.

Anchylostoma duodenale.

New growths of intestine.

Second Day

Tuberculosis.

Methods of infection and spread.

Review of tubercle formation. Chronic solitary tubercles.

Acute miliary tuberculosis.

Specially localized tuberculosis.

Pulmonary—Caseous pneumonia.

Acute disseminated miliary tuberculosis.

Chronic pulmonary tuberculosis. Cavity formation.

Third Day

Pulmonary tuberculosis.

Fibroid phthisis.

Healing of pulmonary tuberculosis.

Laryngeal tuberculosis.

Lymphatic tuberculosis.

Generalized—scrofula, tabes mesenterica.

Tuberculosis of serous cavities.

Tuberculosis of gastro-intestinal tract.

Fourth Day

Tuberculosis of meninges and central nervous system.

Tuberculosis of bone and joints, medullary and epiphyseal forms.

Potts disease—cold abscesses.

Tuberculosis of the skin—lupus vulgaris.

TENTH WEEK

GENERAL OUTLINE

Bacteriology.—Continuation of work on glanders; review of anthrax; consideration of fungi, yeast, streptotrices, sporotrichosis.

Infection and Immunity.—Lectures on Anthrax and Glanders and on Streptotrical infections. Experiments showing methods of diagnosis of glanders in horses, and streptothrix infection in human beings.

Experimental Pathology.—Production of pneumonia and hydrothorax experimentally; obstructive jaundice.

Histopathology and Morbid Anatomy.

First, Second and Third Days

Lesions of leprosy, glanders, actinomycosis, oidiomycosis, streptotrichosis and sporotrichosis; Syphilis.

Fourth Day

Diseases of the liver.

BACTERIOLOGY

First Day

Exercise 22. *Glanders* (b) (Care! very infectious!!).—

The guinea pig infected intraperitoneally shows marked enlargement of the testicles (Strauss reaction); after four to six days the animal usually succumbs to the glanders infection. The postmortem must be conducted with the greatest care. On the peritoneum, between the tunica vaginalis of the testicles, a fibrino-purulent exudate, and, in the parenchymatous organs, numerous small nodules, are seen. The spleen is usually enlarged. Smears are made of the purulent exudate, and the nodules stained with diluted carbol fuchsin or methylene blue (use afterwards a 1 per cent acetic acid plus a few drops of tropaeolin 00). Fine rods of different lengths are seen, resembling diphtheria bacilli, with which they have in common the characteristic segmented staining. Frequently they are also found within leucocytes. From the pus or nodules, glycerin agar tubes and plates are poured. The rest of the tissue is used for sections. Fix in bichloride. The cage in which the animal has been kept must be most thoroughly disinfected.

Exercise 28. Yeasts and Fungi (a).—

In the practical laboratory work, yeasts and moulds are frequently met with as contaminations of plate and tube cultures; on the other hand, these organisms are the cause of various processes of fermentation. Some representatives of this group of these higher organisms are the casual agents in certain diseases. Consequently, it is advisable to understand the general characteristics of these organisms in order to facilitate their recognition.

(a) *Yeasts or Blastomyces*.—Ordinary baker's or brewer's yeast is examined in hanging drop and in stained preparations; yeasts on account of their size are readily distinguished from bacteria. (Note the difference between very large starch grains). The yeasts multiply by a process of budding; on the surface of the cell a minute protuberance develops, which gradually enlarges and forms a daughter cell. The budding may occur at several places, the young cell remaining attached to the mother cell. The protoplasm is homogeneous, or contains granules which stain brownish-violet when treated with iodine (glycogen). Occasionally, a gelatinous envelope (ooglear masses) or capsule is seen. The Gram stain is positive.

The brewer's yeast, being a saccharomyces, forms several endogenous (Ascospores) spores within one cell. Stain these spores by means of the customary methods. Inoculate with pure cultures of saccharomyces cerevisiae and glutinis (torula) gelatin plates, gelatin-stab, potato, glucose, saccharose and lactose-broth in Smith tubes, at room temperature. (14° to 18° C).

Make hanging drop and stained preparations from a culture of the Blastomyces equi. This organism, being a torula, does not produce spores.

(b) *Moulds or Fungi (Hyphomycetes)*.—The attention of the student is directed to the various non-pathogenic moulds. The group of thread fungi or hyphomycetes includes a large number of plants which vary greatly in size, shape and color; the most important of these are the Mucor, Aspergillus, Penicillium, and Oidium as a representative of the fungi imperfecti. Inoculate the bread flasks with the following moulds:

Penicillium glaucum, incubate at room temperature.

Mucor corymbifer, incubate at 37° C for 24 to 36 hours.

Mucor rhizomoriformis, incubate at 37° C for 24 to 36 hours.

Aspergillus niger, incubate at 37° C for 24 to 36 hours.

Aspergillus flavescens, incubate at 37° C for 24 to 36 hours.

Aspergillus fumigatus, incubate at 37° C for 24 to 36 hours.

Pour 2 per cent glucose gelatin plates from a pure culture of Oidium lactis.

Second Day**Exercise 22. *Glanders* (c).—**

On the glycerin agar plates, numerous very small, grayish colonies have grown. They resemble considerably the colonies of the colon bacillus. With high power, small, non-motile, irregularly formed bacilli are seen. They are Gram negative. Sub-cultures are made on agar stab, agar streak, bouillon, gelatin stab and potatoes. Of the last cultures one is kept at 23° C and one at 37° C.

Exercise 28. *Yeast and Fungi* (b).—

The cultures prepared yesterday are examined and studied.

(a) *Yeast*.—Thick, whitish, circular and opaque colonies, which are coarsely granular and slimy, have developed on the gelatin plates. In the stab cultures, the growth is confined to the surface, no liquefaction. On the potato is a thick, raised, white growth. The colonies of the *Sacchromyces glutinis* are pink colored, but of similar consistency and structure as above. Describe. The *S. cerevisiae* has fermented glucose and Saccharose; the *S. glutinis* has left the carbohydrates unchanged.

In microscopic preparations, stained or unstained, the same oval bodies, as seen yesterday, are formed; occasionally they are elongated and give rise to thread-like growths, pseudo-mycelial threads (relation to moulds). The *S. glutinis*, being a wild yeast, does not show these pseudo-mycelia.

(b) *Fungi*.—For the examination of the moulds and their characteristic fruit organs with spores, a portion of the growth is transferred to a watch-glass containing 50 per cent alcohol, to which two drops of ammonium-hydrate have been added. When the growth becomes moist, it should be transferred to a drop of glycerin on a slide. The specimen is thoroughly teased with needles or pins. It is finally covered with a coverglass and examined with low and dry highpower. If the specimen is satisfactory, it may be permanently mounted by placing a ring of asphalt around the edge of the cover-glass.

The vegetative forms consist of threads of filaments (hyphae) which form a meshwork known as the mycelium. The width of the hyphae will vary from 2-5-7 micromillimeters; they may be very short, or as long as $\frac{1}{2}$ -1 inch. Many of the moulds give rise to reproductive cells; eventually spores or conidia are formed. Usually a special thread, the so-called fruit-hyphae, bears on its end the fruit organ. The latter is so characteristic for the different families that it is utilized as a basis of a natural classification.

The *mucor* group is characterized by the presence of spherical saes, or sporangia, on the ends of the vertical fruit hyphae. The sporangium contains spores; the fruit hyphae projects into the sac, forming the so-

called *columella*. The mycelium is colorless. The mucedo also gives rise to *Zygospores* (sexual reproduction) which result by the union of the ends of two mycelial threads.

The *aspergillus* group has no sporangia, but, instead, the ends of the fruit hyphae are enlarged or clubshaped; the enlarged end, or *columella*, is covered with radially arranged bodies—intermediate spore bearers or sterigmata—from which chains, or rows of spores, extend outward. The color of the hyphae and spores varies from black to blueish-gray.

The penicillium has separated and divided fruit hyphae; each branch breaks up into finger-like branches. The end of each branch is not enlarged, but is covered by a number of bottle-shaped, intermediate spore bearers, so-called basidia. Each of these bears a chain or row of spores or conidia.

The *Oidium* does not possess a definite fruit organ. The mycelial threads branch and are usually made up of short, thick cells; chains of large, oval conidia are given off.

Study the cultures of the various moulds (color, odor, etc.) and keep drawings of the fruit organs for comparison. If time permits, inoculate gelatin and carbohydrate broth. Determine the products caused by fermentation.

Exercise 29. *Trichomyces* (*Actinomyces* and *Streptothrix*) (a).—

This group of microorganisms is characterized by its filamentous branching appearance and is supposed to be the intermediate representative, between the bacteria and the moulds. These organisms have been found in air, in water, in many forms of pseudo tuberculosis and various chronic suppurative processes. The most important trichomyces is the *actinomyces bovis*, causing a chronic, specific, purulent, granulating disease in man and animals (particularly in cattle).

Examine the grayish-yellowish pus collected from the abscesses of an actinomycoma ('lumpy jaw') of a bovine animal for small granules, by scraping with the knife over the surface of the diseased tissue. The granules are crushed between a cover-slip, and examined unstained. (Note: (1) hyaline, club-shaped bodies; (2) filaments, and (3) radial striations). Stain cover-slip preparations of crushed granules, by the Gram's, tubercle bacilli and thionin methods. Study sections of actinomycoma (stained with Eosin for five hours at 37° C, and Haematein five minutes, or according to the method of Kühne-Weigert) and pure cultures of the *actinomyces bovis* on coagulated serum. Stain cover-slip preparations and make drawings, etc., of the filaments.

Make microscopic preparations from cultures of various streptothrices (*S. hominis*, *Eppingeri*, *Madurae*) staining by means of Gram's, TB.

thionin and methylene blue methods. Study these preparations and the stock cultures. Inoculate "deep" glucose (2 per cent) agar tubes and potato slices with *Streptothrix hominis*. Incubate at 37° C.

Exercise 30. Pathogenic Yeasts and Fungi (a).—

The literature mentions various local or general affections caused by blastomyces (blastomycosis of the skin, the nervous system and other internal organs, particularly those of the abdomen. Probably some of the cases which have been described as Oidomycosis belong to this group. For this exercise, study stained sections of a guinea pig which has succumbed to a generalized blastomycosis. More important, in America, particularly, are the Oidiomyces. In children, particularly, an affection of the mucous membranes of the mouth, so called "thrush," is caused by *Oidium-albicans*.

Occasionally, smears from diphtheria patients contain the fungus in large quantities. Examine such smears, or prepare slides from the crop of an inoculated pigeon. If time permits, inoculate a rabbit intravenously with 2 c.c. of a heavy suspension of *Oidium* spores suspended in saline. The animal usually succumbs to Oidiomycosis; study the lesions (kidney) and make smears and cultures.

In the vicinity of Chicago several cases of Oidiomycosis, so called blastomycotic dermatitis, have been observed and described. In the tissues of the patient, the parasite appears only as a yeast-like organism; in cultures, typical aerial hyphae develop.

Examine pus from a subcutaneous abscess of a guinea pig inoculated with *Oidium-Americanum* and stain by Gram, and also examine unstained. Prepare cultures on Loeffler's serum. After three to seven days the colonies are examined. The parasite of so termed "Coccidioidal dermatitis" or granuloma found in a fatal disease in the San Joaquin Valley on the Pacific Slope, is also an Oidiomyces with special microscopical appearances in the tissues. Autopsy a rabbit inoculated with "*Oidium Coccidiodes*." Make smears and cultures. Note particularly the double membrane and the endogenous spore formation.

Inoculate 2 agar tubes with *aspergillus flavus* or *niger*.

Exercise 20. *B. Paratyphosus A and B, Dysentery (c).*—

The milk-cultures of *B. enteritidis* and *B. paratyphosus B.* show, after ten days, peptonization and gradual clearing up; the whey becomes yellowish and clear. The *B. Dysentery* and *paratyphosus A.* leave the milk unchanged. The litmus whey of *B. Paratyphosus B.* is very alkaline.

Third Day

Exercise 22. *Glanders* (d).—

The growth on agar and in bouillon is not very characteristic, but more pronounced on gelatin and potato. Kept at 23° C it is extremely poor.

Exercise 29. *Trichomycetes (Streptothrix)* (b).—

Examine the cultures, and, if small colonies are seen, cut out by means of a platinum spatulum—small pieces or slices of agar containing the colonies. Make from a colony impression preparations, and examine stained and unstained.

Exercise 30. *Pathogenic Fungi* (b).—

Diseases caused by fungi (particularly *Aspergillus fumigatus*) are found in human beings only on the cornea and in the respiratory organs: in experimental animals also, interesting processes can be produced by intravenous inoculation.

The following experiments can be done if time permits: (1) Experiment to produce Keratomycesis aspergillina—suspend in a few drops of sterile saline, the spores of the aspergillus grown on agar, as indicated yesterday. Place a few drops of cocaine in the conjunctival sac of a rabbit; then inoculate in the cornea a few drops of the suspension. The needle should be introduced not deeper than about 2 mm., and, if possible, in tangential direction and under slight pressure.

In the course of the following day a turbidity will be noticed. The needle tract is, on account of the injected spores, brownish in color. When examined with the hand lens, fine branches are seen. Finally, the cloudiness of the cornea increases and, at the same time, the grayish portion becomes surrounded by a sickle-shaped zone which represents finally a closed ring of purulent infiltration. Also, a hypopyon will develop, if the virulence of the injected material is sufficient. About the fourth or fifth day the animal is killed, the eyes are fixed in bichloride and, after being hardened, the cornea is excised and imbedded as usual. Sections should be made parallel and vertical to the surface of the cornea. Stain with haematoxylin-eosin. From the needle tract the hyphae extend in all directions. They rarely grow in to the ring of leucocytes, never on the other side of the ring. If the growth of the fungi has been a very slow one, one finds the various threads surrounded by leucocytes.

(2) Experiment to produce pneumonomycesis aspergillina: Inoculate several plates with bread and sterilize, as mentioned above. Sow aspergillus spores on the surface and incubate until the surface is thickly covered with the growth of the fungi. For the experiment, place a pigeon for two hours in a box, or under a bell jar, together with the

Petri dishes containing the pure culture of the aspergillus. Through the action of the wings of the bird, sufficient material is often suspended in the air for inhalation; if not, blow through a piece of glass tubing on the Petri dishes. The next day the pigeon is usually sick; on the second or third day it usually succumbs to the infection. On autopsy, one finds the lungs hepatized, spotted with grayish or yellowish foci. Examine in microscopic preparations by adding 3 per cent potassium hydroxide solution. The fungi threads and the non-germinated spores are very distinct. Fix some tissues in formalin or bichloride. Stain as above.

(3) Experiment to produce aspergillosis by intravenous injections: The result varies considerably, according to the amount of spores used. Prepare a heavy suspension in saline. Dilute one part to such an extent that, with the naked eye, only traces of turbidity are seen. When working with *aspergillus flavus*, inoculate 1 c.c. of this suspension intravenously. When working with *A. fumigatus*, dilute again five to ten times and inoculate the same amount. Another rabbit is inoculated with $\frac{1}{2}$ c.c. of a heavy suspension. This animal generally succumbs in the next twenty-four hours to the infection. Microscopically (unstained, with and without potassium hydroxide), the fungi can be found through the entire animal. All the blood vessels are filled with mycelium. Sections usually give very good pictures of the process.

The other rabbit is killed after forty-eight hours. In the lungs and kidneys, small grayish nodules are seen. Sections, as prepared above, are very instructive.

(4) *Dermatomycosis*: Frequently, fungi are found to cause infection of the skin. The most common types are: *Microspora*, *Trichophyta* and *Achoria*. The scutula (favus discs) of a mouse, artificially infected with *Achorion Quincekeanum*, are teased in a 5 per cent solution of antiformin and examined by low and high power. The variety of forms is noteworthy (spores, mycelia and debris). With a small scab of the material, prepare a hanging-drop or block culture,¹ by using as a medium Sabouraud's maltose agar (see Muir & Ritchie, page 52). Incubate at 35° C.

¹ "*Hanging Block Method*:" Pour melted 4 per cent maltose agar into a Petri dish to the depth of about one-eighth to one-quarter of an inch. Cool this agar and cut from it a block about one-quarter to one-third inch square and of the thickness of the agar layer in the dish. This block has a smooth upper and under surface. Place it, under-surface down, on a slide and protect it from dust. Prepare an emulsion, in sterile water, of the organism to be examined if it has been grown on a solid medium, or use a broth culture; spread the emulsion or broth upon the upper surface of the block as if making an ordinary cover-slip preparation. Place the slide and block in a 37° C incubator for five or ten minutes to dry slightly. Then lay a clean sterile cover-slip on the in-

The epidermic scrapings, or hairs, from a case of human or animal tinea are macerated in a 5 per cent solution of KOH and examined at low and high power. The large ascospores are difficult to distinguish from the chains of spore-like bodies which represent a part of mycelium.

Exercise 31. *Methods for cultivation of anaerobic bacteria (a).—*

The cultivation of pathogenic, anaerobic microorganisms offers great difficulties to the beginner, therefore the ordinary methods should be learned on an organism easy to cultivate. We select for this purpose the butyric acid bacillus, which is very frequently found in milk and soil.

Place about one gram of ordinary soil in a bottle of milk. Boil this mixture for thirty minutes in a water bath (to be counted from the first moment of boiling) and close with a cork stopper as soon as the bottle has cooled down.

By this operation all the vegetative forms of the microorganisms are killed; only the spores of the butyric acid (and hay) bacillus remain alive. The bottle is placed in a dish in the incubator, the top covered with filter paper to prevent any fluid, which may escape when gases are produced in the milk, from spilling over and soiling the incubator. The cork stopper should not be inserted too tightly, as otherwise the risk of breaking the bottle is imminent.

Prepare for the next day: Glucose agar, glucose gelatin and glucose bouillon (1½ per cent glucose) tubes with 15 cm. (for “deep” culture tubes) also with the ordinary quantity; whole milk (thick cream layer); brain emulsion (see later).

Fourth Day

Exercise 21. *Glanders (e).—*

The development on the gelatin is extremely poor. The growth in the stab cultures is filoform and slightly granular, and, on the surface, fine and grayish. On the potato a light brown-yellowish film has formed. Examine this culture again after ten days. The other tubes are very carefully sterilized.

The tissues collected from the autopsy of the guinea pig are imbedded in paraffin—sectioned and stained. The staining process is

oculated surface of the block in close contact with it, avoiding air bubbles. Remove the slide from the lower surface of the block, and invert the cover-slip so that the agar block is uppermost. With a platinum loop run a drop or two of melted agar along each side of the agar block, to seal it to the cover-slip. Place the preparation in the incubator again for five or ten minutes to dry the agar seal. Invert this preparation over a moist chamber and seal the cover-slip in place with white wax or paraffin. Vaseline softens too readily at 37° C, allowing shifting of the cover-slip. The preparation may then be examined at leisure.

difficult, as the tissue is rich in chromatin. It is best to make the sections very thin, which permits the sections to be stained without being brought into contact with alcohol. Place the mounted sections, without passing through xylol, in ten times diluted carbol fuchsin. Take, after half an hour, one hour, one and one-half hours, two and one-half and three hours, respectively, one of the sections; dry carefully; place in xylol and imbed afterwards in neutral balsam.

Another staining method that gives satisfactory results is as follows:

- (1) Xylol, absolute alcohol, etc.; water.
- (2) Stain over night, in the following solution:
Orcein D (Gruebler), 0.1;
Nitric acid (25 per cent), 2.0;
Alcohol (70 per cent), 100.0;
- (3) Rinse, in 70 per cent alcohol;
- (4) Distilled water;
- (5) Stain in Unnas polychrome methylene blue for 1 to 2 hours;
- (6) Distilled water;
- (7) Differentiate in "Glycerin ether mixture;" (Gruebler). Dilute 1 to 2 with water, until the sections are light blue.
- (8) Wash carefully in distilled water;
- (9) Seventy per cent alcohol, absolute alcohol, xylol, balsam.

Exercise 30. *Pathogenic Fungi* (c).—

Examine the hanging drop culture of *Achorion Quinekeanum*. Sporulation, and occasionally the formation of aerial hyphae, has commenced.

Sporothrichosis.—Quite recently a fungus of very interesting morphology and biology has been found to be the cause of chronic localized or generalized affections, which were frequently mistaken for Tuberculosis or Syphilis. In smears prepared with the pus from the sporotrichoma of a rat, only oval, Gram positive spores of sporotrichum "*Scheneki Beurmanni*," are found. Very good preparations are obtained by the Giemsa method.

Inoculate a hanging-block (4 per cent maltose agar) with a small amount of pus, incubate at 25° C.

Exercise 31. *Methods for the Cultivation of Anaerobic Bacteria* (b).—

A repulsive odor in the incubator indicates the growth of *B. butyricus*. The milk has separated into casein and whey. By means of a pipette, a small amount of whey is collected and examined in a hanging drop. Numerous broad, rod-shaped bacilli, perhaps spores, are seen. From this material glucose agar and glucose gelatin plates are poured.

For the cultivation of anaerobic bacteria, the following two rules must be observed: (1) Work quickly, in order to avoid exposure of

the bacilli to oxygen; (2) Use large quantities of the original material, because many anaerobes are at the beginning extremely difficult to cultivate. If once a culture has been obtained successfully, sub-cultures are easy. (3) Use capillary pipettes for transferring material instead of the platinum loop.

The elimination of the oxygen in a culture is most suitably done (a) by absorption with alkaline pyrogallie acid, or (b) by displacing it with hydrogen. The beginner experiments with both. For the isolation of cultures by the pyrogallie acid method, prepare first the reagents and utensils; 50 per cent potassium hydroxide, 20 g. pyrogallie acid in a beaker; one graduate; three glass rods bent in such a manner that they will fit on top of a large Petri dish; and one Novy or special museum or Mason jar (the cover carefully smeared with grease). At the bottom of the Novy jar there should be placed several (16) layers of filter paper, and, on top of this, an empty Petri dish. Then, plates are poured: in the first tube one drop of whey is immersed and in succession the usual dilutions with three loopfuls of material are made. Place the poured plates openly but inverted in the Novy jar, keeping them separated by the glass rods. In the beaker the pyrogallie acid is mixed with 15 cm. of potassium hydroxide solution, carefully shaken, then poured on the filter paper of the Novy jar and the cover closed immediately afterwards as tightly as possible. The pyrogallie acid absorbs the oxygen and, therefore, turns black. The absorption takes place on surfaces and, therefore, filter paper is added. It is advisable to combine this method with the exhaustion of air by a vacuum pump.

For cultivation under hydrogen, the following apparatus are necessary: One Kipp's apparatus, one Novy jar or Museum jar and an air pump. In the Kipp's apparatus, by means of a 25 per cent (1150 c.c. H_2O and 250 H_2SO_4) sulphuric acid solution and small pieces of zinc, hydrogen is produced. The hydrogen, before being introduced into the Novy jar, passes through three "wash" bottles (10 per cent lead nitrate), which have been poured in the same way as described above, are placed in the Novy jar, the apparatus closed, and the rubber tubing from the last wash bottle is connected with the glass spigot in the cover of the Novy jar. Hydrogen is introduced into the jar in form of a slow current; the hydrogen will gradually replace the atmosphere. After twenty minutes collect a small amount of the expelled gas in a test tube. By means of a match ascertain whether it burns slowly (hydrogen), or by explosion (mixture of oxygen and hydrogen). In the latter case, there is still some oxygen present, and more hydrogen is introduced into the jar. As soon as there is absolute certainty that the entire space of the Novy or Museum jar is replaced by hydrogen, the stopper is closed.

It is frequently necessary to exhaust the jar by means of a suction pump. The process of alternate exhaustion and admission of hydrogen may be repeated several times. A very simple, cheap and useful method for the pyrogallie acid process is the following:

On a square glass plate (size 12–13 cm. diameter), a ring or plastilin or “modelin” is formed (30–32 cm. long and 10 mm. thick) corresponding in diameter to the size of the Petri dish to be kept anaerobically. Through this ring a hole (about 3–4 mm.) is pushed by means of a pointed glass rod. On the opposite side of this perforation a glass tubing of the same size connected with a Kipp's apparatus (wash bottles, etc., see above) is passed through the ring. Close to the first mentioned glass rod is placed a small wad of cotton (3 gr.) which, when moistened, can be compressed to a flat disc which is easily fastened to the glass plate by a small piece of plastilin. This cotton is soaked with a few drops of hot water in which 0.2 g. pyrogallie acid has been dissolved. The inoculated Petri dish (add a few drops of methylene blue to the agar before pouring) is inverted and pressed with its edges in the plastilin ring. The modeling clay is carefully smeared around the plate. Open the spigot of the hydrogen generator and no gas should develop. By this procedure, one tests if the plastilin ring is absolutely air-tight. Now remove the glass rod; immediately the gas will enter the dish. After two to three minutes introduce, through the opening in which the glass rod was kept, the needle of a hypodermic syringe and deposit on the piece of cotton 1–2 c.c. of a 50 per cent potassium hydroxide solution. The opening is now closed with plastilin. The gas current should stop immediately. Remove the glass tubing and close at once, with a piece of plastilin, the opening in the ring. Finally make the plastilin wall even around the Petri dish. Incubate at 23° or 37°.

Glucose gelatin plates are kept at a temperature of 23° C and the sugar agar plates are kept in the incubator.

Prepare for the next day one agar slant of *B. avisepticus*.

INFECTION AND IMMUNITY

1. *Differential diagnosis of streptothrix infections.*—If patients are available, the differential diagnosis of streptothrix infections as compared with tubercular infections will be tested.

(a) Case of tuberculosis.

(b) Case of streptothricosis.

Each individual will be given skin tests with acid fast and non-acid fast streptotricin and with old tuberculin.

2. *Diagnosis of glanders in the horse—the ophthalmic test.*—According to the sanitary laws, glanders of horses, being clinically not very char-

acteristic, must be diagnosed by means of the usual serum tests (alexin fixation, agglutination, precipitin test, or conglutination) or, better, by the mallein tests. The thermo-reaction as used with the tuberculin in cattle is very unreliable; the local tests with mallein, on the other hand, give very satisfactory results. The application of a purified, powdered mallein instilled in the conjunctival sac is the simplest method known and the positive results obtained when checked by autopsies and serum tests vary from 94 to 99 per cent. Two horses are selected for the demonstration of the ophthalmic test.

Instill at 5:00 p.m., by means of an eye dropper, two to three drops of a 5 per cent solution of dry mallein in saline, in right conjunctival sac. Before applying the biologic product, examine the eye; the test should not be carried out if any amount of exudate or mucus is discovered in the inner canthus. Distribute the mallein by gently rubbing the eyelids. Take the rectal temperature at the same time.

After 12 hours read results and again take the temperatures of the animal.

A positive reaction is characterized by moderate laceration, pus-like, slimy secretion in the inner canthus of the eye; the conjunctival membranes are deeply reddened; there is frequently a marked edema of the upper and lower lid.

In examining a horse with frank glanders, special care should be taken to avoid infection.

The serum of the horse found infected with glanders should be tested by agglutination or alexin fixation tests. An agglutination reaction of 1:1000 and an alexin fixation with 0.1 c.c. serum are considered indicative of glanders.

EXPERIMENTAL PATHOLOGY

1. Produce obstructive jaundice in a dog. Test coagulation time of blood at intervals subsequently.

2. Test toxicity of bile by injection of varying amounts into peritoneal cavity of rabbit.

HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Leprosy.

Glanders.

Actinomycosis.

Oidiomycosis.

Coccidioides.

Streptothricosis.

Sporothricosis.

Second Day**Syphilis.**

The primary lesion.

The secondary lesions.

The tertiary lesions.

Hereditary syphilis. Colles' law.

Characteristic lesions in skin, nasopharynx, bone, liver, lungs, etc.

Vascular syphilis—Aortitis—Heubner's endarteritis.

Syphilis of the nervous system.

Syphilitic meningitis.

Gummata.**General paralysis.****Tabes.****Third Day****Review of chronic granulomata.****Fourth Day****Liver.**

Developmental anomalies.

Congenital cystic liver.

Anomalies of position and form.

Hepatoptosis.

Infection with *B. Welchii*.

Atrophies.

Senile—brown atrophy.

Necrosis—focal.

Degeneration and infiltration.

Hydropic — Parenchymatous — fatty—amyloid — Glycogen —
Alcoholic.

Acute yellow atrophy.

Red atrophy.

Eclampsia.**Pigmentations.**

Icterus—icterus neonatorum.

Hemachromatosis—malarial pigmentations.

Circulatory disturbances.

Hyperemia.

Passive congestion—cyanotic atrophy and induration.

Thrombosis and embolism.

Leukemia.

ELEVENTH WEEK

GENERAL OUTLINE

Bacteriology.—Chicken cholera; bubonic plague.

Smears and cultures of *B. diphtheriae*; involution forms in old bacterial cultures. Cultures of *B. tetani* and *B. oedematis maligni*.

Infection and Immunity.—Lectures on plague and the first lecture on toxins and antitoxins.

Experimental Pathology.—Experimental production of pancreatic diabetes. Renal glycosuria.

Asphyxial glycosuria; and acute hemorrhagic pancreatitis.

Histopathology and Morbid Anatomy.—Diseases of the liver, gall bladder, and pancreas. Diphtheria.

BACTERIOLOGY

First Day

Exercise 30. *Pathogenic Fungi* (d).—

Examine the hanging block culture of *Sporothrix*. Long mycelial threads, with numerous laterally inserted spores, are seen.

Study sections of a typical sporotrichoma and compare with a tuberculoma.

Exercise 31. *Methods for the cultivation of anaerobic bacteria* (c).—

On the agar plates, usually from the outside, a growth is noticed. The Novy jar is opened carefully in manner to be demonstrated. The plates are taken out, closed by their respective covers, and examined. On the first plate numerous gray colonies, which have produced gas, are seen. On the second and third plates the colonies are less frequent and more uniformly distributed; their size is about one to two millimeters. At sixty times enlargement, the colonies appear to be granulated with irregular eroded margins. In the stained cover-slip preparation, long bacilli are seen. From several of such colonies, with a long platinum wire, stab cultures in glucose agar and glucose gelatin (in "deep" tubes) are prepared.

Exercise 32. *Fowl cholera* (a).—

One rabbit is inoculated subcutaneously, or one pigeon intramuscularly, with one loopful of a culture of *B. aviseptious*.

Exercise 33. *Plague* (a).—

The cultures (in broth and agar) and the organ smears, for this exercise, have been sterilized with formaldehyde gas, owing to their great danger. Make hanging drop preparations and smears stained with very diluted carbol fuchsin, gentian violet, and by Gram. Note the polar staining and the streptobacilli forms in broth.

The typical lesions, caused by this bacillus, in guinea pigs and rats are studied on preserved specimens.

Exercise 34. *Diphtheria* (a).—

The material for this exercise is obtained from a patient suffering from diphtheria. By means of a cotton swab, the diseased part of the pharynx is touched. The material collected on it, is used for the study. Cover-slip preparations are stained with methylene blue and by Gram. Diphtheria bacilli are rod shaped, straight or slightly curved, thicker and larger than the typhoid bacillus. In the middle, or at one end, they are occasionally slightly thicker; at the ends, pointed or clubbed, rarely branched. They often show a dotted or beaded appearance; little granules are present and deeply stained. They are Gram positive only when decolorized very quickly. The other bacteria present in the material vary considerably in numbers.

Cultures are prepared only on Loeffler's serum:

1. By smearing the swab over 3 plates (a, b, c).
2. Soaking the swab in the water of condensation and then smearing it over the slanted serum surface. The swab remains in the tube and, after six hours, the water of condensation is distributed over the serum again by tilting it carefully ("smear tube"). Incubate.

In case no swabs are available, use pure culture to obtain a subculture on Loeffler's serum.

To obtain pseudo diphtheria bacilli, collect, by means of a cotton swab, material from the conjunctiva and smear it also on Loeffler's serum.

Prepare for the next day: agar slant cultures of *B. typhosus*, anthrax, fowl cholera, prodigiousus, pyocyaneus, and vibrio Metchnikovi.

Second Day**Exercise 31. *Methods for the cultivation of anaerobic bacteria* (d).—**

In the cultures prepared yesterday, a filiform growth with gas pearls is observed, along the needle track. The growth does not reach the surface. For the preparation of glucose bouillon, brain, and milk cultures, it is advisable to boil the tubes for fifteen minutes to expel the oxygen and inoculate them (when cooled down) with a drop of agar stab culture. The tubes are kept under hydrogen, or with pyrogallie acid, in the Novy jar or Buchner's tube, as previously explained. The

cotton plug of the tube should not be inserted too tightly. A similar method can be used, that of stratifying a few centimeters of liquid paraffin on the sugar bouillon and boiling both fluids together for sterilization. Afterwards, the tubes are inoculated through the paraffin. Such tubes need not necessarily be kept anaerobically, but this method is necessary for certain anaerobes. Keep all the tubes in the incubator.

Exercise 32. *Fowl Cholera* (b).—

The animals inoculated with the culture are dead. A post-mortem is performed in the same way as described for the anthrax mouse. Cover slips are stained and prepared in the same way as these described. Several of these preparations are fixed, not by heating, but by keeping them for five minutes in ether-alcohol (ether and absolute alcohol, equal parts). When dried they are stained with Giemsa solution as above. In the preparations fixed over the flame, small bacilli, often lying in pairs, are noted. In the preparations fixed with ether-alcohol and stained with Giemsa, they retained the stain only at the ends (bipolar). They are Gram negative.

From the heart blood or the organs, agar and gelatin plates are poured. Small pieces of the organs are fixed in mercury bichloride.

Exercise 34. *Diphtheria* (b).—

The diphtheria cultures are best examined after six hours' incubation; the colonies are small, but the other bacteria associated in the material have not yet grown.

The first plate is used as a "smearplate," the others as "isolation plates," and the tube as a "smear tube." First examine the "isolation plate."

After 24 hours the diphtheria colonies are usually large, circular discs, opaque-whitish, yet not like porcelain; when the colonies are very numerous and rather confluent, they are very distinct.

Prepare hanging drop preparations and stain ordinary smears with methylene blue. They are non-motile and, in stained preparations, appear as large, club-shaped rods. Particularly characteristic is the position of each organism to the other. Occasionally they form an angle or appear like spread fingers. The beading is very distinct.

Stain one preparation with Neisser's stain.

(1) Fix, by heat or alcohol.

(2) Stain the films for a few seconds with the following solution:

(a) Methylene blue 1 part, absolute alcohol 50 parts; Glacial acetic acid 50 parts; distilled water 1,000 parts.

(b) Crystal violet (Hoechst) 1 part; absolute alcohol 10 parts; distilled water 300 parts.

Mix solution (a) two parts and solution (b) one part.

- (3) Wash in water;
- (4) Apply Lugol's solution plus 1 per cent lactic acid for 2-3 seconds (not longer).
- (5) Wash in water;
- (6) Stain in cresoidin solution (1 to 300) for a few seconds. (The cresoidin should be dissolved in warm water and the solution filtered). Wash in water. Dry and mount.

Examine such preparations with a dry, high power lens. In the round, stained bacilli, numerous dark-blue metachromatic granules are seen.

To obtain an absolutely pure culture, it is advisable to plate out a colony—which has been recognized microscopically as consisting of diphtheria bacilli—on glycerin agar plates. Surface smears on glycerin agar will give, occasionally, better results.

When the diphtheria bacilli are very few, they are easily found, together with other organisms, by means of the "smear culture." The bacterial growth is removed with a strong wire and distributed in a large drop of water on the slide. When stained with methylene blue, or after Neisser, the organisms are readily seen.

The colonies of the pseudo diphtheria bacilli are usually small. Stained with carbol fuchsin they also appear irregular in thickness but are mostly pointed at the ends. Their relative positions to each other is very characteristic and of diagnostic importance. They are parallel. With Neisser's stain, no granules can be demonstrated.

Exercise 35. Involution forms of Bacteria (a).—

Bacteria of the same species have generally the same appearance but, under certain conditions, they grow out to peculiarly swollen, aberrant, degenerated forms. Such "Involution Forms" can readily be produced by adding lithium chloride to the culture media.

Add to twenty-one tubes of nutritive agar, containing 10 c.c. agar, such an amount of a 10 per cent aqueous solution of LiCl, that the medium contains one, two and three per cent of LiCl. Sterilize for one-half hour in running steam. Slant the tubes and inoculate each organism, prepared yesterday, on three tubes. Also prepare a culture of the *B. Prodigiosus* on agar and incubate at 37° C.

Third Day

Exercise 31. Methods for the cultivation of anaerobic bacteria (e).—

In the bouillon a fine flocculent turbidity and gas bubbles are observed; the milk is changed in the same manner as in the original material. The jars are opened and the usual strong odor of butyric acid is noted. The cultures are examined microscopically.

In the gelatin stab cultures, along the needle track, small colonies are seen. The growth is more distinctly anaerobic than in the agar stab cultures.

Exercise 32. *Fowl cholera* (c).—

On the agar plates small colonies have developed. The superficial ones are round and gray yellowish, in transparent light they are bluish or opalescent. The colonies in the depths are gray yellow, wedge shaped or round. Large colonies are only formed by condensed water or dirt. At sixty times' enlargement, the superficial ones are yellowish, transparent and finely granular. On the gelatin plates no colonies are visible to the naked eye. Under the microscope they resemble those just described for the agar.

Examine in hanging drop: The bacilli are very small and non-motile. Prepare stained preparations; the bacilli appear as in the organs. They are very seldom bipolar. Spores are not formed. Prepare several sub-cultures, particularly gelatin stab, gelatin streak, agar stab, agar streak, bouillon cultures and shake cultures in sugar agar.

Wash the organs fixed in HgCl_2 in running water.

Exercise 34. *Diphtheria* (c).—

On the glycerin agar plates, the diphtheria bacilli have grown singly or mixed with other bacteria. The colonies are transparent, light, with net-like inside structure and with very distinctly granular margin. Those in the depths are round or wedge-shaped, darker and granular. Some of the colonies, which have microscopically been identified as those of diphtheria bacilli, are transplanted under the microscope, stained and examined. If they are truly diphtheria bacilli, the following sub-cultures are best prepared: agar stab and streak; gelatin stab and Loeffler's serum.

Should the isolation offer considerable difficulties, a diphtheria colony of the first serum tube is inoculated on another serum slant and, on the following day, plates are prepared from this last tube.

Exercise 35. *Involution forms of bacteria* (b).—

The cultures prepared yesterday are examined in stained coverslip preparations (carbol fuchsin). Compare the organisms with mounted permanent specimens. The bacilli have grown on the lithium chloride agar to long filaments of irregular thickness, some are spherical, yeast, bottle or sausage shaped. If such forms are rare, keep the cultures at incubator temperature and examine on the following days, for fourteen days.

Beautiful involution forms are found in old cultures. It is, therefore, best to examine the cultures which have been prepared previously; particularly the anthrax and the vibrio cultures.

Exercise 36. *Tetanus* (a).—

Inoculate a mouse, at the tip of the tail, with soil which has been artificially infected with tetanus spores. Inoculation is made subcutaneously and not directly in the middle of the root of the tail, as the local tetanus occurring in the mouse is of diagnostic importance.

Fourth Day

Exercise 32. *Fowl cholera* (d).—

The colonies on the agar plates are only slightly enlarged as are also the colonies on the agar streak and stab cultures. In gelatin cultures the growth is rather minute; somewhat better in the bouillon cultures (slight turbidity). In the carbohydrate agar stab cultures no gas is formed. All the cultures can be destroyed after an agar stab and slant culture have been prepared. These are sealed the next day and kept.

In the glucose gelatin stab cultures the growth and the liquefaction have progressed. The jar with the glucose gelatin plates is opened. Gas has been produced. The colonies of the *B. butyricus* consist, at sixty times' enlargement, of filaments; they liquefy the gelatin. They are examined microscopically and sub-cultures in glucose agar and sugar gelatin in "deep" tubes are made. In addition to these bacteria, other spore-bearing microorganisms may be found on some of the plates.

Exercise 34. *Diphtheria* (d).—

The surface cultures on the glycerin agar plates show toothed and ragged margins; microscopically, branched forms are frequent. They are more numerous on the following days. The growth in the agar stab and agar streak cultures is poor, even more so on the gelatin stab culture; on Loeffler's serum it is abundant. From one of these cultures, three bouillon tubes are inoculated: by holding the tube as vertical as possible, the margin of the surface is inoculated to obtain, if possible, the formation of a pellicle. To ensure this formation float a drop of oil on the broth. (Sterilize oil in a beaker at 150° C).

Exercise 36. *Tetanus* (b).—

The mouse shows a local tetanus which usually affects the limb nearest to the seat of infection. In the next few days the affection extends over the other muscles.

Exercise 37. Malignant edema and blackleg (a).—

For the study of pathogenic anaerobes the outline of Hibler is of great advantage. The examination of material suspected to contain anaerobes is conducted as follows:

1. *Microscopic examinations* of tissues (muscle juice), in small animals serous membranes (peritoneum, liver surface).
Use diluted carbol fuchsin; Gram's method, and examine moist preparation in Angol's solution for "granuloses."
2. *Preservation of material for further tests*: Dried or moist under H₂ or CO₂.
3. *Animal experiment*: for purification of anaerobe (usually guinea pigs).
4. *Enrichment*: In rabbit blood or serum or milk with H. for B. tetani.
5. *Culture in deep tubes*: Boil for one-half hour before use.
 - (a) plain agar 2 per cent (plus 0.5 per cent).
 - (b) plain gelatin (10 per cent) plus Peptone (1 per cent) plus 0.6 per cent NaCl. Rarely an addition of sodium indigo sulphate or sodium formate is needed. For some organisms 0.5 to 1.5 per cent glucose or litmus solution, 1 to 5 per cent, is added.
 - (c) Brain emulsion (Katechol). One part minced brain substance boiled for one hour with $\frac{1}{3}$ to $\frac{1}{4}$ distilled water; tubed and sterilized at 100° C for three consecutive days.
 - (d) Milk with cream (sterilize); keep at 56° to 60° C when not sterilized.
6. *Test spore resistance*: in brain media and spore formation in alkaline potato media.
7. *Pathogenicity*: inoculate subcutaneously on the back.

For further details consult the notes.

Inoculate a guinea pig in a skin pocket at the abdomen, with a small amount of soil infected artificially with malignant oedema bacilli, or with a piece of tissue containing the B. oedematis maligni.

Another guinea pig is inoculated, with ground muscle substance which contains the blackleg bacillus or the Ghon—Sachs bacillus. Add to the emulsion to be inoculated, two to three drops of lactic acid to prevent phagocytosis.

EXPERIMENTAL PATHOLOGY

1. Diabetes. Produced by complete removal of pancreas in dog. Follow sugar output quantitatively.

2. Renal glycosuria—25 gm. phlorizin dissolved in 5 c.c. warm sterile water and injected in rabbit.

3. Asphyxial glycosuria is produced by injection of $2\frac{1}{2}$ c.c.. Four per cent solution of morphin. Also observe Cheyne-Stokes type of respiration.

4. Puncture glycosuria.—Trephine just behind the occipital protuberance of a rabbit and introduce puncture knife, pushing it in a line joining the external auditory meati till it strikes the basilar bone. Examine urine in 2 hours.

5. Acute hemorrhagic pancreatitis produced by injection of 3 c.c. of dog's bile into pancreatic duct of same animal.

First Day

Liver—Inflammatory conditions.

Acute—in septicaemic conditions; of metastatic origin from intestines and appendix; of biliary origin.

Chronic inflammations.

Hepatic cirrhosis.

Atrophic (Lannee); Hypertrophic (Hanot); Alcoholic.

Tuberculosis of the liver.

Syphilis of the liver.

Perihepatitis chronica hyperplastica (iceing liver).

Second Day

Gall bladder.

Inflammatory conditions—Cholecystitis and cholangitis.

Catarrhal; Purulent; Ulcerative—perforation—pericholecystic abscess; Empyema of gall bladder.

Chronic cholecystitis.

Pericholic adhesions.

Gall stones.

Hydrops of gall bladder.

New growths of liver and gall bladder.

Third Day

Pancreas.

Fatty infiltration.

Parenchymatous and fatty degeneration.

Acute pancreatitis.

Acute hemorrhagic pancreatitis.

Chronic intestinal pancreatitis.

Islands of Langerhans—hyalin atrophy.

Diabetes mellitus—Bronzed diabetes.

Pancreatic concretions.

Fourth Day

Diphtheria.

The local lesions.

Pulmonary complications.

Lymphatic apparatus and spleen.

Kidney lesions—Adrenal lesions; Nervous lesions.

TWELFTH WEEK**GENERAL OUTLINE**

Bacteriology.—Work on fowl cholera, diphtheria, malignant oedema, blackleg, and tetanus continued. Flagella stains. Study of *B. botulinus* and bacteria of the proteus group.

Infection and Immunity.—Two lectures on toxins and antitoxins and one on anaphylaxis. Experiments to demonstrate serum anaphylaxis. The action of diphtheria antitoxin on its toxin. Tetanus toxin and antitoxin.

Experimental Pathology.—The production of various types of nephropathy.

Histopathology and Morbid Anatomy.—Diseases of the genito-urinary system.

BACTERIOLOGY**First Day****Exercise 32. Fowl cholera (e).—**

The cultures of fowl cholera are sealed and kept at low temperature.

The organs are sectioned in the way previously described and stained with fuchsin after Gram.

Exercise 34. Diphtheria (e).—

The film on the agar streak culture is thin and grey. The surface growth of the stab culture is similar, but slight. The growth along the needle-track does not offer any peculiarities. On the agar plates, the margin of the colonies is even more ragged than yesterday. Make impression-preparations. The bacilli appear distinctly club-shaped; branching is common. On the gelatin stab, the growth is like a string of pearls; scanty, no liquefaction; in the broth it is abundant and in form of a clumpy conglomeration, which forms a sediment. Frequently a surface pellicle has formed. Such cultures are frequently more virulent, the formation of the toxin is increased by the oxygen. In microscopic preparations with high power, one finds the bacilli to be pointed, less club-shaped than on Loeffler's serum: they are also in small clusters.

The bouillon cultures of the diphtheria bacilli are distinguishable from those of the pseudodiphtheria bacillus by the formation of acids and toxins. Determine the amount of acid by titrating with one-fortieth normal sodium hydroxide solution. The diphtheria bacilli usually pro-

duce an amount of acid which equals 1 to 1.55 c.c. of one-fortieth normal sodium hydroxide solution. The test for virulence is conducted by inoculating one guinea pig with 0.2 and one with 1 c.c. of broth. The inoculation is made subcutaneously at the abdomen, not directly in the mid-line.

Exercise 37. Malignant oedema and blackleg (b).—

No. 1. Malignant oedema: The guinea pig, inoculated with malignant oedema usually succumbs to this disease 1 or 2 days after the inoculation. At the seat of injection a broad, extensive swelling has formed; gas formation is rarely observed. As the distribution of the bacilli does not take place by the blood stream, but by the lymph stream, only a few bacilli are to be expected in the internal organs, and therefore a careful examination of the local oedema must be made. At the seat of the injection, many different types of bacteria are present, but the farther away from this point the examination is made, the purer the culture of oedema bacilli will be found. To avoid contamination of the material, the postmortem of the animal (after careful disinfection of the skin with alcohol) should be conducted in such a way that the skin is split from the margin of the oedema to its center. A sterile knife is then used and the skin removed from the abdomen carefully. A subcutaneous tissue and the muscles show serous, hemorrhagic infiltration. A small piece is removed for examination and the carcass is covered with moist cotton, as the bacilli multiply after death. In examining the hanging drop, large motile bacilli and various spore forms the size of anthrax bacilli and containing rods and cocci, are seen. Long filaments and chains have formed on the peritoneal lining. Make liver impression preparations. Cover-slip preparations are stained with fuchsin and by Gram. The latter is only positive when the decolorization is conducted quickly. Cultures on glucose agar and glucose gelatin plates, with several loopfuls of material, are prepared and kept anaerobically in hydrogen or with pyrogallie acid, etc. Furthermore, shake cultures are prepared in "deep tubes" as follows: 4 to 6 tubes of glucose agar or gelatin are boiled for 15 minutes (to expel oxygen). The first tube is inoculated with several loopfuls of oedema fluid, then from the first to the second, from the second to the third, dilutions are made. The material is not plated as usual, but at once coagulated upright, standing in ice water, forming so-called "deep tubes." The heart blood is inoculated in brain emulsion and blood broth (see next paragraph).

The balance of the material is fixed in formalin and examined in sections, as usual.

(b) Blackleg: The postmortem lesions of the guinea pig are similar to those just described, with the exception that the muscle tissue may

be more severely involved. The bacilli are also frequently found in the spleen and on the surface of the liver. Examination of these organs should, therefore, be made in addition to the examination of the muscles. Microscopically, the spore bearing bacilli are very characteristic, as so-called "closteridium forms" are produced. The bacilli stain readily by Gram. Cultures in glucose agar, glucose gelatin and "deep tubes" are prepared, as described above. Heart blood in brain emulsion, and blood broth.

Exercise 36. *Tetanus* (c).—

The mouse inoculated with tetanus material usually dies after four days. In addition to the local tetanus, a characteristic position is usually noted. The front legs are close to the body and the hind legs stretched (the sea-lion position). The bacilli produce, at the seat of the infection, a most intensive toxin, but they do not disseminate in the body. Therefore, an examination of the internal organs is not necessary. After removal of the skin, microscopic smears are prepared from the seat of inoculation. Organisms are found similar in appearance to the anthrax bacilli, thinner and spore bearing. Occasionally the spores are characteristically localized at the end of the bacillus (so-called "drumstick" forms). They stain by Gram. Many other bacteria may be found.

The isolation of the tetanus bacillus is, on account of the presence of other contaminating microorganisms, comparatively difficult. In order to eliminate at least a part of these contaminating bacteria, as much material as possible is scraped from the seat of infection and placed into a test tube which contains a very small amount of sterilized saline solution. This test tube is kept for an hour in a water bath at 65° C. From this mixture, glucose agar and glucose gelatine plates are poured. Furthermore, coagulate a few centimeters of blood, collected from the ear vein of a rabbit at high temperature, and inoculate this medium with the same material as above, as stab culture. Keep the glucose agar, the gelatin plates and the blood under hydrogen at 23° C.

Second Day

Exercise 34. *Diphtheria* (f).—

Should one of the infected guinea pigs be dead, perform an autopsy (see findings next day). Frequently one or both animals are sick; they are apathetic, the body temperature is sub-normal, at the seat of the inoculation a soft oedema extends to the regional inguinal lymphnode.

Exercise 36. *Tetanus* (d).—

From the blood tubes the characteristic odor of anaerobes is noticed. They are heated for an hour at 65° C, and then plates are poured which are also placed anaerobically.

Exercise 37. Malignant oedema and blackleg (c).—

(a) In the same way that *B. butyricus* may vary considerably, oedema bacilli may show differences from those usually described in text books in the following way: The colonies on the glucose agar are the size of 1 to 2 mm., the superficial ones are round; the colonies in the depth are wedge shaped. At sixty times' enlargement the margin is finely granulated or shows numerous branches. A few bacilli are just visible. Other bacilli and cocci may also have grown.

If no oedema bacilli are found on the plates, the internal organs or the guinea pig, autopsied yesterday, are examined again. They are now usually found in large numbers and practically pure cultures can be made. Plates are again poured from these organs.

The cultures of the oedema bacilli are examined in a hanging drop and in stained cover slip preparations. From a few colonies, stab cultures in glucose agar and glucose gelatin ("deep tubes") are prepared; bouillon, milk (in Smith tubes), streak cultures on Loeffler's serum and brain emulsion are made and kept anaerobically.

In the "deep tube" cultures, numerous colonies have developed in the depth of the medium, and produced considerable gas. To make sub-cultures from such colonies, the bulk is removed. This is best done as follows: After careful disinfection of the tube, the glass is broken with sterile instruments in a sterile dish, or the test tube is heated from top to bottom until the part of the agar, adherent to the glass wall, is liquefied; the agar plug can then be thrown out into a sterile dish. Inoculate slanted agar from one of the colonies by means of the needle.

(b) The changes in the culture media and the growth observed are similar to those described above. Sub-cultures on glucose agar, bouillon, milk, brain emulsion, are also made and, particularly, blood bouillon is inoculated. The blood bouillon is prepared by drawing, with a sterile needle, the blood from the jugular vein of a horse, and letting it run into sterile bouillon tubes (about $\frac{1}{3}$ of the bulk). Let the blood coagulate in the bouillon and then incubate for forty-eight hours (to test the sterility of such prepared tubes). Such tubes are inoculated with material from an isolated *B. chauvei* colony.

Exercise 38. Flagella stain (a).—

Preparation of the mordant for the flagella stain according to Bunge. The following solutions are prepared: Tannic acid, 5 g. dissolved in 50 c.c. of water; then, 10 c.c. iron sulphate solution (1:2) and 2.5 c.c. saturated alcoholic fuchsin solution are added.

For the Zettnow stain: (a) Prepare antimony mordant: Dissolve 2 g. antimony tartrate in 40 c.c. of water: 10 g. tannic acid in 200 c.c. of water. Heat the latter at 50° to 60° C, then add to the tannic acid

solution 36 to 37 c.c. of the antimony solution, and heat until the deposit formed is completely dissolved again. Then a small quantity is placed in a test tube and put into cold water. The sample tested should soon become turbid. If it remains clear, another c.c. of antimony solution is added. If it becomes milky, a small amount of solid tannic acid is added. When heated from 75° to 80° C, the mordant becomes absolutely clear, and when cooled, no precipitate should occur.

(b) Ethylamine or ammonia silver solution: Prepare a saturated, watery solution of silver sulphate (2 to 3 grams in 200 c.c. distilled water). Pour a small amount in a test tube, add the same amount of water and, drop by drop, a 33 per cent ethylamine solution is added, until the deposit formed is completely dissolved again. *Preparation:* Place in a test tube a small amount of meat and water in such quantities that the water does not cover the meat entirely. Keep this preparation at 23° C. *Prepare* agar slants of the *B. typhosus* and *Vibrio Metchnikovi*, obtain liquid manure with large spirilla.

Third Day

Exercise 34. *Diphtheria* (g).—

At least one of the inoculated guinea pigs has succumbed. In performing the autopsy, the seat of inoculation is particularly exposed. A marked hemorrhagic oedema is noticed. In the pleura and peritoneal cavities a small amount of exudate; the adrenals are enlarged and hemorrhagic; general hyperemia of the other organs and, particularly, the anterior portions of the small intestines.

Microscopically, only a few organisms are found at the seat of the inoculation. In the other organs, and particularly in the pleural exudate, they are extremely scarce, or entirely absent.

If the animal does not succumb to the small doses inoculated, the seat of the inoculation becomes necrotic after the lapse of a few days, and the dead tissue is sloughed. The animals may succumb, even after weeks or months, to the infection.

Exercise 36. *Tetanus* (e).—

On the agar plates, as a sequel to the heating, only spore-bearing bacilli have grown. The tetanus colonies are very small and rare. Suspicious colonies are examined in smears and, if consisting of drumstick forms, mice are inoculated for tests. As the toxin is concentrated, the animals succumb more quickly than when they have been inoculated with soil material. Make sub-cultures in glucose broth, agar stab, milk and brain emulsion, gelatine stab or shake colonies.

Exercise 37. Malignant oedema and blackleg (d).—

(a) In the glucose agar stab cultures, abundant growth is observed along the needle track, but this growth never reaches the surface. The gas formation is very marked. In glucose gelatin the growth is poor. The three days' old gelatin plates are also examined. The colonies are gray and have a diameter of $\frac{1}{2}$ to 1 mm. At low power, the appearance of the gelatin cultures is similar to that of the *B. Butyricus*. Numerous other microörganisms have grown. Make stab cultures. The milk is coagulated and slight gas formation is seen. During the following days, the coagulum usually becomes peptonized (watery, yellowish fluid with floating cream). In the brain emulsion a blackish coloration associated with a repulsive odor is noticeable. The blood broth is dark and frothy.

One of the agar stab cultures is removed, as described, and microscopically examined. If possible, stain also for flagella. Flagella are very numerous and peritrichous. Stain for spores, if they are seen in the hanging drop.

(b) The growth is the same as found in (a) with the exception that the milk is not yet coagulated the brain emulsion remains pinkish.

Exercise 39. Bacteria of the proteus group (a).—

The water in which the raw meat was immersed yesterday is examined in the hanging drop and in stained cover slip preparations. Four gelatin and four agar plates are poured as usual.

Exercise 38. Flagella stain (b).—

It is essential to have absolutely clean coverslips, otherwise the flagella stain will not be successful. They are best cleaned with benzine and a piece of silk. When once cleaned, they should not be touched further with the fingers, but with clean forceps which have been treated with benzine. The simplest staining method is according to Bunge. The mordant to be used should be at least one day old.

Prepare, from the pure cultures, very thin suspensions in distilled water. Place one loopful of water on three coverslips. A trace of culture is inoculated in the first, from there to the second, and from there to the third drop. Let the smears become air-dry, and place some of the mordant on the coverslips. Heat the coverslips (without boiling) for two minutes. (Consult instructor). Rinse in water and re-stain if necessary, with carbol fuchsin. Rinse again in water, dry and mount in cedar oil. Flagella are stained distinctly and most easily on spirilla. The typhoid bacilli show flagella over the entire body (peritrichous). The bacilli usually appear larger. The preparations are not always stained uniformly. If the stain has not been successful, then first investigate

whether the technical directions have been followed carefully. Frequently the coverslips are not cleaned properly, or the mordant is too fresh or too old, or the bacterial suspension is too heavy.

If this flagella stain is successful, stain a few preparations according to Zettnow. Heat a small amount of antimony mordant in an embryo dish with glass cover (over a boiling water bath or hot iron plate). In the meantime, prepare the dilutions on the coverslips by transferring a small amount of the bacteria from one drop to the other, as described above. Place also, in the different drops, one or two loopfuls of osmic acid and spread the drp carefully over the surface of the coverslip. Afterwards, fix the coverslips by passing them twice through the flame. Such coverslips are ready to be floated on the surface of the hot antimony mordant. They remain on the solution from five to seven minutes, then they are removed and cooled until the fluid on the coverslip becomes turbid. They are then rinsed in water and are, from now on, handled only with forceps. Three to four drops of silver solution are placed on the coverslip and heated until the fluid smokes. The edges of the coverslips appear black and not simply yellowish. Rinse again, dry, and examine in cedar oil or Canada balsam. The bacteria and the flagella are black, on an absolutely clear background. The stain is unsuccessful when the coverslips have not been cleaned properly, or the different parts of the stain have not been rinsed sufficiently or heated long enough.

Exercise 40. *B. Botulinus* (a).—

B. Botulinus—the cause of a form of meat poisoning, with characteristic nervous symptoms—is an anaerobe. Prepare, from stock cultures, glucose agar and glucose gelatin plates. Incubate, under anaerobic conditions, at 23° C. Prepare also glucose broth, glucose agar-stab and glucose gelatin-stab cultures under anaerobic conditions. Keep at room temperature, as many strains of *B. Botulinus* do not grow at 37° C.

Fourth Day

Exercise 37. *Malignant oedema and blackleg* (e).—

In the glucose agar stab cultures, a marked growth has taken place. The jar with the bouillon and serum cultures is opened, and an intense odor is noticed. The bouillon is turbid and has a heavy layer at the bottom of the tube. The serum is liquefied, producing holes in the media.

A re-inoculation with the pure cultures can be made by mixing the bacteria with sterilized quartz sand or sterilized soil.

(b) Blackleg: The cultures of the blackleg bacillus are examined in a similar manner. In the inoculated blood bouillon tubes, a formation

of gas is very distinctly visible, and the accumulated gas bubbles, on top of the fluid bulk, resemble charged water. The milk is uniformly coagulated and does not become changed in the next few days.

Exercise 39. *Bacteria of the proteus group (b).—*

On the gelatin plates, different varieties of colonies have grown. At sixty times' enlargement, those with filament-like growth are most noticeable. With high power it is shown that such colonies consist of bacilli. From an absolutely isolated colony, agar stab, agar streak, potato, milk and bouillon cultures are prepared.

On the agar plates none of these characteristic colonies are visible. Still, the bacteria are there, only the colonies on agar are less characteristic than on gelatin.

Exercise 40. *B. Botulinus (b).—*

The appearance of the *Botulinus* cultures are similar to those of the tetanus colonies on agar plates. The colonies on the gelatin plates have liquefied; in the liquefied zone at 60 times' enlargement granules or filaments are seen. In the hanging-drop and stained preparations, large, plump rods, motile and with polar spore, are seen. In the glucose agar and gelatin stab, distinct emanation of gas. The growth is not very characteristic. In broth, uniform turbidity with sediment and gas bubbles. The odor is the same as in other anaerobes.

Inoculate 0.001 c.c. of broth subcutaneously on the hind leg of a mouse. After two hours, this extremity is usually paralyzed—the paralysis progresses rapidly to the other leg. The legs are dragged when the animal makes an attempt to escape. Finally, the muscles of the fore-legs become paralyzed and, after a few hours, the animal succumbs.

Guinea pigs, rabbits, cats and pigeons can be used for this experiment. In addition to the paralysis, marked dilatation of the pupils is noted.

INFECTION AND IMMUNITY

1. The action of diphtheria antitoxin on its toxin:

A double series of mixtures of a sample of diphtheria toxin and of diphtheria antitoxin, of which the L.+ dose and antitoxic units are known, is made in Rosenau syringes, the sterile needles of which are temporarily plugged with sterile vaseline, as follows:

1. Diphtheria toxin (L + dose in 1 c.c. NaCl) + 2 units antitoxin.
2. Diphtheria toxin (L + dose) + Normal horse serum, corresponding to antitoxin in No. 1.
3. Diphtheria toxin ($\frac{3}{4}$ L + dose) + 1 unit antitoxin in 1 c.c.

Stir mixtures in syringes by rolling and, after standing for 15 minutes, inject each mixture subcutaneously in a normal guinea pig, weighing from 200 to 250 grams. Note local lesion daily, and autopsy carefully—if the animals die. Keep survivors for at least three weeks and note symptoms of toxon paralysis.

2. Anaphylaxis to Horse Serum in Guinea pigs.—

Five guinea pigs were given 0.01 c.c. of sterile normal horse serum three weeks previously (sensitization). They are now treated as follows, with control normal animals.

- (a) Sensitized G.P. No. 1. Given 0.5 c.c. horse serum in jugular vein.
- (b) Sensitized G.P. No. 2. Given 5 c.c. horse serum in Peritoneum.
- (c) Normal G.P. No. 1. Given 0.5 c.c. horse serum in jugular vein.
- (d) Normal G.P. No. 2. Given 5 c.c. horse serum in intraperitoneally.

EXPERIMENTAL PATHOLOGY

Experimental Nephritis (to be continued in the following week):

1. Hydronephrosis—Ligation of ureter in rabbit. Autopsy after 3 weeks.
2. Pyonephrosis. Inject into pelvis of kidney 1 c.c. 24-hour culture of *B. coli*.
3. Glomerular nephropathy. Inject 0.7 mg. uranium nitrate into renal artery of rabbit.
4. Interstitial nephritis. Inject 1 to 2 c.c. 0.05 to 0.02 per cent watery solution of iodine very slowly into the renal artery.
5. Tubular nephropathy. Inject 1 c.c. of a 2 per cent solution of potassium chromate into the renal artery.
6. Acute nephropathy with edema. Inject 1 mg. uranium nitrate subcutaneously and give 50 c.c. water daily by stomach tube.
7. Mercuric nephropathy. Inject subcutaneously 10 c.c. 1-1000 HgCl₂ daily.
8. Arsenic nephropathy. Inject 10 mg. potassium arsenate. Examine urine and make autopsy at end of hour.

HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Genito-urinary system.

Congenital anomalies.

Kidneys—Absence of one or both; hypoplasia—horseshoe—congenital cystic; Adrenal(?) inclusions; Fetal lobulation; Anomalies of ureters; Bladder—ectophron; Testes; Hypo- and hypospadias; Ovaries; Uterus; Vagina; Pseudohermaphroditism.

Kidney—Gross and microscopic anatomy of—vascular arrangement of; functions of.

Misplacements (Nephroptosis).

Hydronephrosis.

Regeneration and hypertrophy of kidney.

Second Day

Kidneys: Infections of; Abscess; pyelonephritis.

Degenerations—Parenchymatous, fatty, colloid, amyloid, senile.

Albuminuria and casts.

Acute diffuse nephropathy.

Acute interstitial nephropathy.

Third Day

Acute glomerular Nephropathy.

Subacute and chronic parenchymatous nephritides.

Chronic interstitial nephropathies. Arteriosclerosis of kidneys.

Fourth Day

Uraemia.

Uric acid infarcts.

Bilirubin crystals.

Urinary calculi in kidneys.

Tuberculosis of kidneys.

New growths of kidney and adrenals.

THIRTEENTH WEEK

GENERAL OUTLINE

Bacteriology.—Continuation of study of malignant oedema, *B. proteus*. Beginning study of bacteria of influenza and of whooping cough. Staining and cultivation of spirochetes. Bacteria present in air and soil. Fusiform bacteria.

Infection and Immunity.—Visit to the Cutter Analytic Laboratory to inspect method of commercial production of vaccines and specific sera, particularly of antitoxins.

Experimental Pathology.—Experimental Nephritis (continued).

Histopathology and Morbid Anatomy.—

First and Second Days

Diseases of genito-urinary system (continued).

Third Day

Malaria and diseases of unknown etiology; small pox, rabies, etc.

Fourth Day

Diseases of internal secretion.

Introduction to study of tumors.

BACTERIOLOGY

First Day

Exercise 37. *Malignant Oedema* (f).—

In the gelatin stab cultures, liquefaction has taken place, and in the depth of the liquefied zone a flocculent deposit is seen. The cultures may be destroyed.

Exercise 39. *Bacteria of the Proteus Group* (c).—

Gelatin is liquefied by many bacilli of this group. Frequently, from the needle track, numerous branches extend into the medium. The agar and potato cultures are not characteristic. The bouillon is uniformly turbid—the milk coagulated.

Exercise 41. *Influenza and Whooping Cough.*—

The organisms causing these diseases are found chiefly in the respiratory passages. If fresh material can be obtained, it is used for the exercise; otherwise pure cultures are studied in a similar manner.

The tenacious, glassy sputum is poured in a Petri dish; search for small pus particles, which are used for smears. The microscopic preparations are best stained with a very (forty times) diluted carbol fuchsin solution, for five to ten minutes. The bacilli of influenza are minute, oval rods, in length about the same as the width of the anthrax bacillus. In typical cases they are present in large quantities, particularly between the leucocytes. Usually such other organisms as pneumococci, streptococci, etc., are seen.

For cultivation, blood agar is used. This culture medium can be prepared by adding defibrinated blood in the quantity of one-tenth to liquefied agar, in the same manner as has been explained for the preparation of serum agar. The method described by Bordet (Muir & Ritchie, page 44) can be used. A very simple method, which gives very satisfactory results, is as follows:

Slanted agar tubes are smeared on the surface with a few loops of human blood; the blood is collected from a finger; disinfect the skin by rubbing it with 60 per cent alcohol; puncture with a flamed needle and enlarge the escaping blood-drop by pressing on the finger or by shaking the arm. Instead of human blood, the blood of rabbits or pigeons can be used, the latter being collected from the vein of a wing after disinfection of the seat of puncture. The blood is carefully rubbed with a platinum loop into the culture media. A pus particle of the sputum is carefully distributed in several tubes without flaming the loop when transferring from one tube to another.

The control tube of blood agar is infected with streptococci, also a few plain agar tubes are inoculated with the influenza sputum.

The bacillus pertussis is of the same size as the Influenza Bacillus and gives the same staining reactions. Make some subcultures from a pure stock tube by using, as culture medium, the blood agar medium of Bordet & Gengou (Muir & Ritchie, page 44).

Exercise 42. *Spirochaetes* (a).—

The spirochaetes, the cause of the so-called "*Spirochaetoses*" (not spirilloses), represent an independent group of unicellular, flagellated-like organisms; they are characterized by slender, eel—or spiral shaped forms. The systematic position is not entirely established; some forms undoubtedly belong to the animal protista, inasmuch as they show certain correlation (Plastic nature of the body and polymorphism) to the "*trypanosomes*" and allied forms ("*proflagellates*"). On the other hand, some, on account of the nuclear structure and comparatively small size, are closely related to the bacteria and represent, perhaps, the transitional phylogenetic stage between flagellates and bacteria.

Representatives of the spirochaetes are widely distributed, both as saprophytes and as parasites. The latter are usually separated into:

(a) *Endo and ecto-saprophytes or parasites*: present in mouth cavity, respiratory tract, genital tract, superficial ulcers, intestinal tract, stools, etc., of man and animals.

(b) *Blood parasites*: causing various forms of relapsing fevers, febrile anemias in man, birds and mammals.

(c) *Tissue-parasites*: The most important representatives are *Treponema pallida* (Syphilis) and *Tr. pertenue* (Yaws).

The body of the spirochaetes is very slender, having a circular, transverse section, and not band shaped, as originally described. The length varies from 2 or 5 to 150 to 500 microns, the thickness from 0.5 to 3.0 microns; it is not advisable to use the size for the identification of the species, as variations in very broad limits are the rule. The body of spirochaetes always show more or less regular convolutions or twists, which are characteristic for many species. In some cases (*Treponema pallida*) the twists are true spiral convolutions around the ideal longitudinal axis, but in many forms aside from true spirals the existence of serpiginous curves in one plane have been demonstrated (*Spir. Obermeieri* Duttoni, etc.). The motion of the spirochaetes is very complicated. (a) flexion; (b) rotation, and (c) movement forward and backward, depending on various factors (see experiment on *spirochaeta buccalis*).

Structure and Development of the Spirochaetes.—

In testing the behavior of spirochaetes towards Trypsin, Saponin, NaOH, sodium taurocholate, etc., it is found that the larger species, like *Cristispira*, possess a distinct sheath or periplast. For small spirochaetes a distinct membraneous covering of the protoplasmic body has not definitely been proven. For the oyster spirochaetes, a so-called *undulating membrane*, which winds spirally around the organism from end to end, has been demonstrated; in this membrane distinct fibrils (myonemes) are seen; many spirochaetes, when macerated, show marginal flagella which are interpreted as representing a part of the periplast. In comparing this membrane with the organ of trypanosoma or haematoproteus, the impression is gained that the marginal flagellum is an indication of the development of the undulating membrane, and that the gradual disappearance of the plasmalamella finally leads to the attachment of the marginal flagellum to the protoplasmic body. The demonstration of elastic fibers is, therefore, essential to separate spirochaetes from the inflexible spirilles. Flagella are probably not found on the spirochaetes; the descriptions of such appendages refer to artefacts produced by macerating or staining methods. The inside structure of the spirochaetes also shows differences between the *Cristispira* and the small

spirochaetes, but further investigations are needed to settle the existing dispute. The nucleus is probably distributed in form of "chromidia" (*nuclear bands*) over the entire body, or is condensed in form of a permanent, rod-shaped nucleus (*Cristispira*). The mode of division of spirochaetes takes place in one group by *longitudinal*, and in another (*Molluse-spirochaetes*) by *transverse division*. Sexually differentiated forms have been described, but it has not yet been proven satisfactorily that there exists a process of conjugation. Considerable attention has been paid to the breaking up of spirochaetes, in minute masses of chromatic granules, which are considered to be developmental stages of the parasites.

Many of the most important spirochaetes have quite recently been cultivated. The specific immunity reactions do not correspond with those known for bacteria.

I. Cristispira balbiani (Certes).—

This very large organism is placed first, as most of the features explained above can very readily be found. The spirochaete is found in the anterior part of the oyster's digestive tract, particularly in the so-called crystalline style. Incise the closing muscles of the bivalve, reflect the valves, and, by making a longitudinal incision through the oyster, expose the stomach and the crystalline style. Collect, with a platinum loop or pipette, material for living and stained preparations.

(a) *Living Preparations*: Mix the material in a small drop of saline, cover immediately with a thin coverglass and surround it with vaseline or yellow wax. (Many spirochaetes are strictly anaerobic). This preparation can be studied with an ordinary microscope by making free use of the diaphragm and lowering the condenser. The best illumination is obtained by using a dark field illuminator or Burri's Indian ink method. The handling of this delicate apparatus, together with demonstration, will be a part of this study.

The indian ink preparations are made by the student personally: One drop of indian ink (Pelikantusche von Gröbler, Leipzig), diluted 5 to 10 times with distilled water* is carefully mixed with one drop of material, uniformly (like blood smears) over a fat-free slide. When dry, examine with the oil immersion lens. The spirochaetes appear as fine white spirals on a brown or black-brownish background, similar to the dark field illumination.

* The original ink is, distributed already diluted in test-tubes ($\frac{3}{4}$ c.c.), tightly closed with a cotton plug and sterilized for an hour on two to three successive days. Let the indian ink sediment for two to three weeks. Without shaking the tube, collect the ink by means of sterile capillary pipettes.

(b) *Permanent preparations*: Very thin smears, after being air-dried, are fixed for ten minutes in absolute alcohol or methyl alcohol. Or, better still, the material is smeared on coverslips which have been previously exposed to vapors of osmic acid. After the preparation, the film is again exposed, for a few seconds, to osmic acid. The smears are stained according to the following methods:

- (1) Giemsa Method: for one to twenty-four hours.
- (2) With Loeffler's mordant (see later).

For the demonstration of the undulating membrane, the material is distributed in a drop of distilled water, then smeared out, dried, and fixed. By using distilled water, a slight maceration is obtained.

The *Cristispira balbiani* is slender, filiform, with rounded ends, frequently band-like and rather flatly curved. The length varies from 26 to 180 microns; the width is 0.5 to 3 microns. The motion is similar to that of a trypanosome, and consists of rotation simultaneous with forward or backward movement. Independent bending movements are observed. The number and the form of the spirals are constantly changed during these actions. Most of these movements are brought about by the actively contracting periplast.

The marginal flagellum of the so-called undulating membrane, which can be seen in the unstained and stained preparations, does not end with a free flagellum. Some forms have no undulating membrane. The marginal flagellum breaks up very readily in a number of fine fibrils and breaks off from the body.

The protoplasmatic body is divided into numerous, separate cells, which contain the chromatin but never form a definite nucleus. Occasionally, a more or less complete helix of chromatin is formed in the inner protoplasm. The multiplication takes place, as a rule, by transverse division; longitudinal division has also been observed.

(2) *The spirochaetes of the mouth cavity.*—

In the mouth cavity of man, as well as of animals, two or more species of spirochaetes are found. The two most commonly found are: *Sp. Buccalis* and *Sp. Dentium*.

For the examination: collect, by means of a flamed platinum needle or loop, a small amount of mucus from the gums, or from a carious tooth, and prepare smears as described under (1), (a) and (b). If sufficient material is at hand, the following method for permanent specimens can be recommended:

Place in a drop of 1 per cent osmic acid, several loops of material, and from these distribute it, in thin smears, on coverslips which are dried as rapidly as possible in the flame of the Bunsen burner.

Stain by Giemsa, Leishman¹ and with Loeffler's mordant.² In using the latter treat the preparation for about two minutes with the freshly filtered mordant by gently heating the coverslip high over the flame. Wash very carefully in water, then stain with carbol fuchsin by heating until steam arises. Rinse carefully in water. Dry and imbed in cedar-oil. All preparations should be studied and drawn in living and in stained conditions.

The small *Sp. Dentium* can be cultivated by the following method: In a few c.c. of serum bouillon a dilution is made with the material collected from the mouth cavity. By means of a long platinum needle, inoculate serum or ascites agar (one part serum and two parts agar, cooled to 42° C) and mix carefully. With the same needle make two other dilutions in the agar to obtain as isolated colonies as possible. Solidify the tubes immediately in cold water. Cover the solidified agar with paraffin oil and place the tubes in a Novy jar under anaerobic conditions. Incubate at 37° C.

The large *Sp. buccalis* shows flat, irregular spirals and is either rounded or pointed at both ends. The length of the thread-like body varies from 12 to 20 mm.; the width 0.5 to 1 mm. During motion, the spirals change tremendously. The motility is the same as found for *Sp. balbianii*.

The motility can be temporarily suspended by narcotizing the spirochaetes with quinine or with another chemical. The following experiment should be conducted to demonstrate the temporary inflexibility of the mouth spirochaetes:

Examine by dark field illumination, the mucus from the gums, for spirochaetes. If actively motile forms are present, allow a formamint tablet slowly to dissolve in the mouth, make another preparation and note the gradual recovery of the paralyzed rigid spirochaetes.

¹ Leishman's stain is applied as follows:

- (1) Prepare films; dry; but do not fix.
- (2) Flood the unfixed film with stain (0.15 g. of powder, 10 c.c. methyl alcohol puriss, Merck); allow to act.
- (3) Add double the volume of distilled water to the stain on the film and mix with glass rod or platinum loop.
- (4) Allow this diluted stain to act five minutes.
- (5) Wash off with distilled water.
- (6) Leave some water on film for 30 seconds to intensify the color contrasts.
- (7) Dry and mount.

² Loeffler's Solution:

Aqueous solution of tannic acid (20 gr. in 100 c.c boiling distilled water)	100 c.c.
Cold saturated solution of ferrous sulphate	50 c.c.
Saturated alcohol solution of Woolschwarz	10 c.c.

In stained preparations, the organism is fixed in various positions of movements. An undulating membrane is rarely seen. Frequently, flagella-like protoplasmatic filaments are seen at one or both ends, particularly when stained with Loeffler's mordant.

No nucleus can be distinguished. When stained by Giemsa, the entire body is reddish blue (uniform mixture of chromidia with protoplasm). Division is extremely rarely seen. Longitudinal division, with separation of the daughter cells, opening out at an angle of 180 degrees, often gives the impression of transverse division. Frequently, during separation, flagella-like periplast fibrils are produced.

The *Sp. Dentium* is much smaller and finer than the *buccalis*. Length is, as a rule, 4 to 10 μ i. and $\frac{1}{4}$ μ i. in thickness. This form shows constantly, also during motion, regular curves. The motility takes place by rotation and very rarely by bending movement. When at rest, some refractile places can be seen moving over the protoplasmic body and therefore the existence of an undulating membrane is highly probable.

Frequently, towards the ends of the body, the spirals become lower and a gradual pointing of the spirochaetes is noticed. Flagella-like attachments are also seen. Nothing is known about the conditions of the nucleus. Generally speaking, the form is characterized by a remarkable constancy of the spirals.

Multiplication probably takes place by longitudinal division and, for this reason, dividing stages in form of a "V" or a "Y," are frequently observed.

Exercise 43. *Germs in the atmosphere and earth (a).—*

This exercise is done particularly to demonstrate the possible contaminations in bacteriological work. Three agar plates and three gelatin plates are poured in Petri dishes, then the cover is removed and they are left open at different places. One agar plate is placed where the air is clean and pure, another one in a dusty place, both for about an hour. The two other plates are covered in very thin layers with fine earth dust. Potatoes cut in slices and sterilized are covered with hay dust. The sterilization of the potato slices is not absolutely necessary. These potato slices are put into a glass dish which is covered at the bottom with filter paper soaked in mercury bichloride solution. Incubate one set at 37 degrees, one at 23 degrees.

Second Day

Exercise 41. *Influenza and whooping cough (b).—*

The colonies of the influenza bacillus appear on the blood agar as very small, entirely transparent dots; on the ordinary agar they are absent. Examine the cultures with a hand-lens in transparent light (hold

against the window). In comparison with the Streptococci, which are turbid, they are entirely transparent. In addition to the Influenza Bacilli, other organisms have grown.

The Influenza Bacilli are examined in a hanging drop and in stained coverslip preparations. They appear again as very small rods, occasionally grown to long filaments; they are non-motile and Gram negative. Make subcultures from pure colonies on ordinary agar and blood agar.

The Bacillus of Whooping Cough shows a scanty growth, is less transparent than in the case of Influenza Bacillus. Microscopically, the organisms are similar but less pleomorphic than the Influenza Bacillus.

Exercise 42. *Spirochaetes* (b).—

(3) *Sp. Gallinarum*: In geese and chickens a spirochaete has been found to be the cause of a septicemia which is characterized by an acute or a chronic course. The parasite is transmitted by ticks belonging to the group of argas. (*A. miniatus*, *A. persicus*).

The spirochaete is best studied in blood smears made from an infected bird, where the parasites are usually extremely numerous.

Technique of blood examination: The feathers are removed from the inside of the wing; the skin carefully disinfected with alcohol and then, by pricking a superficial vein, a drop of blood is collected by means of a pipette or clean coverslip. This blood may be examined either as a fresh film (especially by dark field) or as a dried and stained film.

***Fresh Films*:—**

Method 1. Take up the drop of blood on the center of a coverslip and drop it at once onto a slide; surround the coverslip immediately with vaseline or with paraffin.

Method 2. Cement a coverglass to a slide with paraffin wax or vaseline as will be shown; the drop of blood is added near the edge and will spread by capillary attraction.

***Dried Films*:—**

Take up a drop of blood on the under edge of one end of a slide; the drop should then be brought into contact with another slide near its end and after the drop has properly spread by capillary attraction the first slide should be gently pushed along the surface of the second, holding it at an angle of about 45 degrees. An acute angle will give a thin film, an obtuse angle will give a thick one. After being dried, the films are fixed by immersing for ten minutes in methyl alcohol or absolute alcohol or absolute alcohol-ether, equal parts. The preparations are stained either with diluted carbol fuchsin or by Giemsa or with Loeffler's mordant.

Between the nucleated blood cells, delicate, flexible, long, wavy spirals, with twenty turns, are seen. The length is usually from 16 to 30 μ . They are much narrower than a common bacillus. They are very actively motile and preserve their motility at the ordinary room temperature for many days. For details, read Noguchi, *Journal of Experimental Medicine*, 1912.

(4) *Sp. Obermeieri* and *Sp. Duttoni*:—

These spirochaetes are found in European Relapsing Fever and in African Recurrent Fever. They are especially found during the febrile paroxysms, at which time they may be present in large quantities. For our studies we use infected mice or rats. The blood is collected, for the examinations, by clipping the ends of the tails, making fresh films for dark field examinations and dried films for permanent specimens. The material is fixed and stained, as already described under Chapters 1 and 3. In addition, the following methods are recommended as being of diagnostic importance:

(a) Stain with very diluted Manson's methylene blue (100 c.c. boiling water, 5 g. borax and methylene blue pur. med, Hoechst 2 g.). The staining solution should be old, otherwise no polychromatic staining reaction is obtained. In a well stained specimen, the spirochaetes are dark blue, the red blood cells greenish.

(b) Giemsa's solution: stain for three-quarters of an hour. The moist fixing method, which will be discussed later, can be successfully applied.

(c) Staining in a thick drop: For the demonstration of a very few parasites, it is advisable to concentrate the material for examination and, instead of smearing the blood drop over the entire slide, place several drops on very clean slides and let them become air dried. A very thick film can also be used. The specimens, after being thoroughly air dried, are fixed, but stained directly with Manson's or with Giemsa's solution (stain for one hour); rinse the slide in a glass of water; do not flush it under the faucet; let it dry in the air and not between filter-paper.

(d) By adding a few drops of a one per cent (1 per cent) solution of acetic acid to a drop of blood and centrifugalizing the material, an enrichment can be obtained. Make preparations from the sediment.

Make an autopsy of a mouse which has succumbed to the infection. Note particularly the anatomical lesions in the spleen, kidneys, etc. (Care! infection!)

In fresh preparations, the spirochaetes are recognized as delicate, flexible spirals, with the motions described previously for the other spirochaetes. The bending is extremely active. The cessation of the movements is not an absolute proof of the death of the spirochaetes.

Usually, by heating the slide, the active motility is restored. No undulating membrane has been conclusively demonstrated. Frequently, the spirochaetes are found in clumps.

In stained preparations they are fixed in various forms of motion. A differentiation into protoplasm and nucleus is occasionally possible. Granular-like inclusions have been described. The development takes place by longitudinal division. So-called granule shading has been observed and been interpreted as rest, or cyst, forms.

Transmitters of Spirochaete Duttoni:—

The spirochaete *Duttoni* is generally transmitted by the tick *Ornithodoros maubati* (Murray). In connection with the Exercise on Spirochaetes it is advisable to study specimens of the tick; detailed information can be found.

General shape of the body: ovoid, somewhat wider behind than in front; yellowish-brownish in color when young, greenish-brown when adult. Integument studded with small tubercles. On the dorsal surface: three pairs of grooves running obliquely downwards and inwards towards posterior end. Above the bases of the legs: a longitudinal supra-coxal groove. On the ventral surface there is a deep pre-anal groove joining the supra-coxal grooves laterally; behind the anus: three pairs of longitudinal grooves.

There are no eyes present. Stigma: semilunar in front above the supra-coxal groove. Fourth leg: one and a half times as long as the first; tibiae and tarsi: of first three pairs with three teeth on the upper side.

Exercise 43. *Germs in atmosphere and earth (b).—*

On all the plates and potatoes, colonies have developed. On those kept at 23 degrees they are small. Examine about six type colonies which are particularly numerous. The examination is done by the naked eye, at sixty times' enlargement, hanging drop and stained preparations (carbol fuchsin, carbol thionine, methylene blue, Gram). Make an agar streak and milk culture, and perhaps make agar plates if the colonies are suspected of being mixed, and inoculate bouillon and potatoes.

Note in a tabulated form for each colony examined:

Number of the colonies.

Appearance of the colonies.

Motility.

Staining reactions.

Growths on the different media.

In this exercise take particular care that no two colonies, which are lying close together on the plate, are examined and transplanted.

Search particularly for bacteria belonging to the group of the micrococci (irregular, grape-like clumps); sarcina (divisions in three dimensions) colonies usually yellow; short bacilli; colonies with root-like growth; long bacilli; colonies folded and wrinkled, particularly on potato; microscopically spore bearing bacilli (*bacillus mesentericus*); colonies of fungi, their irregular star-like branches extending, after a few days' incubation, over the greater part of the medium, etc.

Third Day

Exercise 42. *Spirochaetes* (c).—

(4) *Treponema Pallida*: *Treponema pallida*, the cause of syphilis, resembles in its morphology the *Sp. dentium* and can be readily distinguished from the *Sp. Refringens* with which the *treponema pallida* is frequently associated. The *Sp. Refringens* resembles the *Sp. buccalis*. For the examination of this parasite, obtain coverslip preparations from a fresh syphilitic process (juice smears). In the course the material will be obtained from a syphilitic orchitis of a rabbit. Make living preparations for dark field illumination and also for the Burri Method, as previously described. For the dilution of the material, use only ascitic fluid.

Stained preparations.—To obtain good results, only very thin smears on absolutely clean slides or coverslips are necessary. After being properly air dried, fix as usual in alcohol and stain afterwards with Giemsa, for about one hour. The quick staining method with Giemsa cannot be recommended, as staining deposits are often obtained. These can be avoided by using distilled water after having air dried the smears. The staining method would then be as follows:

(1) Air dry the smears; place for a few minutes in distilled water; air dry again very carefully.

(2) Place the coverglass in a Petri dish and cover with Giemsa solution acetone mixture, to cover the slide entirely. Let the staining fluid act for one or two minutes.

(3) Add 15 c.c of strongly alkaline water (1 to 2 drops of 1 per cent sodium hydroxide solution to 50 c.c. water). Mix carefully and stain for 10 minutes. Rinse in water.

Stain preparations also with fresh aniline water gentian violet solution for 24 hours.

Very intense staining is obtained by using Loeffler's mordant, as described above. It is advisable to use smears from absolutely fresh material only. Impression preparations from tissues are never satisfactory.

Sections will be distributed for the demonstration of spirochaetes in the tissues. The material has been stained according to the method of Levaditi. The technique is briefly as follows:

(1) Small tissue pieces (2 mm. thickness) are fixed in formalin (1 part plus 9 parts water) for 24 hours.

(2) Place in 96 per cent alcohol for 12 to 16 hours.

(3) Wash in distilled water until the discs sink to the bottom; change water several times.

(4) Impregnate in the following freshly prepared solutions: 1 per cent silver nitrate solution; 90 c.c.

Piridin pure: 10 c.c.

The tissue pieces remain in this solution at room temperature for 2 to 3 hours, and afterwards for 3 to 5 hours, at a temperature of 45 to 50° C (dark bottle with glass stopper).

(5) Rinse quickly in 10 per cent piridin solution.

(6) Reduction for a few hours, or over night, in the following freshly prepared solution:

Four per cent pyrogallie acid solution: 90 c.c.

Acetone pure: 10 c.c.

Add to 85 c.c. of this mixture, 15 c.c. piridin.

(7) Rinse in water, alcohol scale, xylol, imbed in paraffin. Make sections of 5 to 10 mic. thickness.

The spirochaetes appear black, on a light brownish ground.

With regard to the cultivation, consult Noguchi: *Journal of Experimental Medicine* 1911.

The *treponema pallida* resembles the *Sp. Dentium*, on account of the fineness, poor light refraction and affinity for stains. The short spirals and the constancy of the form during motion are also very characteristic. The length varies from 10 to 20 mic. The thickness is even less than that of the *Sp. Dentium*. From the *Sp. Dentium* the *treponema pallida* is distinguishable by the ratio of the length to the depth of the windings and the form and size of the angle of the windings; the ratio for the *pallida* being 1: 1 or 1: 1.5, whereas in *Sp. Dentium* the ratio is 1:1.5.

Flagella-like protoplasmic appendages are frequently seen on *treponema pallida*. They are proportionately very thick. The multiplication takes place by longitudinal division. First, the flagella-like protoplasm portions divide, the body becomes somewhat thickened, and in a few seconds afterwards it divides in its entire length; whereas the two daughter cells separate only by apparent transverse division.

The real longitudinal division is, on account of the very rapid occurrence, rarely caught in stained preparations, and in living preparations is observed only with difficulty.

(5) *Treponema pertenue*: The examination of *Tr. pertenue*, the cause of yaws, is conducted in absolutely the same matter as just explained for *Tr. pallida*. Study the differences in the anatomical lesions of rabbits inoculated with *T. pallida* and *pertenue*.

Exercise 41. *Influenza and Whooping Cough (c).—*

On the blood agar tubes the same growth has taken place as above described. On the agar without blood, no growth is noticed. Should there be colonies, make microscopic preparations and determine if the colony consists of small rods. If this is the case, transfer again to plain agar, as it is highly possible that traces of blood, which have enabled the growth, have been transmitted from the initial colony.

Exercise 43. *Germ of atmosphere and earth (c).—*

The cultures prepared yesterday are carefully examined. Make microscopic preparations from milk cultures, particularly when the fluid is not changed. It is advisable to fix such slides or coverslips in ether-alcohol (equal parts) for a few minutes. The fat becomes dissolved and the films fixed to the glass. After removal, evaporate the ether and alcohol and then stain as usual.

The cultures are kept at the same temperature for further examination.

Fourth Day

Exercise 43. *Germ in atmosphere and earth (d).—*

The examination of the germs is continued. The cultures made on the second day are examined and, by means of text books, certain types are identified. New colonies which have appeared are examined in the same way as described above, namely, microscopically and by means of cultures. On several plates, fungi have grown. Microscopic preparations are best used to compare the size of the filaments with that of the bacilli, and the size of the spores with that of the cocci.

Exercise 44. *Fusiform bacilli.—*

Frequently associated with spirochaetes, particularly in the so-called Plaut-Vincent's angina, are found thin rods which are straight or slightly curved or tapered at the extremities, showing, in the central portions, unstained points and granules.

Make stained preparations, particularly by Giemsa, from material which has been collected from a case of angina. Examine pure cultures which have been grown under anaerobic conditions. Compare the organisms with spirochaetes and note the difference.

Exercise 21. *Tuberculosis (d).—*

The serum culture of the tubercle bacilli is thicker. On the glycerin agar tubes, small scales have grown, which increase in the following weeks, until the entire culture shows a folded granular film. On the glycerin broth a fairly thick pellicle has developed.

Experimental Pathology.—Continuation of work of last week.
Histopathology and Morbid Anatomy.—

First Day

Bladder—acute infections; chronic infections; tuberculosis; Calculi.
Prostate—Hypertrophy; acute infections; chronic infections; tuberculosis.
Urethritis.
Balanitis—syphilis; soft chancre.
New growths of bladder, prostate, and testes.

Second Day

Female genitalia.

Vulva—infections of—Post partum lesions—fistulae.
Vagina—acute infections—chronic infections.
Uterus—misplacement of.
Endometritis—acute—chronic, tuberculosis.
Salpingitis—acute, chronic, hydrosalpinx, tuberculosis.
Oöphoritis.
Post partum infections.
Pelvic abscess.
Chronic pelvic inflammatory diseases.
Senile changes in genitalia.
New growths in female genital organs.

Third Day

Malaria.

Lesions produced by filterable viruses—Rabies, smallpox, etc.

Fourth Day

Organs of internal secretion.

Hypo—and Thyperthyroidism.
Cretinism and myxedema.
Goitre—simple and exophthalmic.
Hypophysis—hypo—and hyperpituitism.
Gigantism and acromegaly.
Adrenals.
Addison's disease.
Ovaries and testes.

Introduction to the study of tumors.

Hypertrophy. Metaplasia. Heterotopia—Heteroplasia. Anaplasia
or Reversionary metaplasia—Keloid formation.

FOURTEENTH WEEK

GENERAL OUTLINE

Protozoology.—Study of amebae; sarcosporidia; flagellates. Trypanosoma lewisi.

Infection and Immunity.—Lectures on infectious diseases of unknown causation; chemotherapy; experimental syphilis and the Wassermann reaction.

Class demonstration of the Wassermann reaction for syphilis.

Histopathology and Morbid Anatomy.—Tumors.

PROTOZOOLOGY

First Day

Lecture on structure and development of protozoa.

Biology of transmitters.

Classification of the Protozoa.

General technique used for the examination of protozoa.

In all examinations of protozoa, the study of the unstained specimens is absolutely essential. By properly using the diaphragm and the condenser, even the finest structure of the protozoan cell and the character of motility, nutrition, etc., can be properly studied.

Examine, if possible, in the medium in which the parasite is found, or use saline solution. Place on a slide a drop of the material to be examined and cover it with a coverslip which has previously been edged by vaseline. The hanging-drop method can also be used.

When examining very active protozoa, the addition of a small amount of gelatin to the medium will reduce the motility and permit a better examination.

Always examine, first, with a high power lens before using the oil immersion.

The examination is occasionally considerably assisted by adding a drop of a very diluted neutral red solution (1:800 to 10,000) to the medium.

For the study of finer structures, particularly of the nucleus, fixed and properly stained specimens in smears or sections can be used.

The organs are either impressed or smeared by means of forceps on coverslips or slides, and immediately afterwards placed in the hot fixing solution. Blood or secretions are smeared on slides in the following manner: a drop of blood is taken on the surface of a slide near one

end. The edge of another slide is brought in contact with this drop, which then spreads out so as to fill the angle between the two slides along the whole extent of the line of contact. On pushing the upper slide towards the other end of the lower slide a film of blood will be left behind. The thickness of the film is easily regulated for if the angle between the two slides is acute, the film left behind will be very thin. If the angle be nearly at right angle, a thick film will be left. An angle of about 45 degrees gives the desired thickness. Such films are either air-dried or, better, fixed in bichloride or other fixing solution mentioned below. Intestinal contents, stools, etc., are best smeared by means of a flamed platinum wire on coverslips or slides.

In all cases, the smears should be very quickly made to prevent drying.

All preparations should be fixed moist and should be treated as moist specimens in a similar manner to sections. The coverslips or slides are floated on the fixing fluid and afterwards inverted.

For fixation, use (a) Bichloride alcohol; (Schaudinn); saturated aqueous mercury bichloride (7 per cent in boiling distilled water) two parts; absolute alcohol, 1 part.

or—

Flemming's solution

(b) Two per cent osmic acid, 1 part; 1 per cent chromic acid, 1 part; 1 per cent aqueous acetic acid, 2 parts; distilled water, 13 parts.

Smears are fixed in a few minutes, in these solutions, particularly when used hot (70° C); small pieces of tissues remain for one-half hour to six hours. The smears fixed in bichloride, except those to be stained by Giemsa, are washed for half an hour in 60 per cent alcohol to which sufficient tincture of iodine is added to color it a port wine yellow, then placed in fresh 60 per cent, then 70 per cent and 80 per cent alcohol where they can be left until ready for staining. Tissues are placed in 70 per cent, then 80 per cent alcohol, where they may be kept until they are wanted for imbedding in paraffin. The sections obtained are to be run through iodine alcohol, as above, before staining.

Smears fixed in Flemming's solution are washed in distilled water for 10 minutes and then passed through plain alcohol.

For the staining of protozoa in smears and sections, the following methods are used: (1) Delafield's hematoxylin: stain in very diluted solution one-half hour to twenty-four hours, rinse in tap water; examine under microscope; if over-stained, differentiate (constantly controlling under the microscope) with acid alcohol (0.1 c.c. hydrochloric acid in 100 c.c. 70 per cent alcohol). When the nuclei are very distinct, wash in running tap water for 15 minutes, pass through 70 per cent alcohol, 95

per cent alcohol, 100 per cent alcohol; xylol alcohol into xylol (one minute each); mount in balsam or cedar-oil.

(2) Borax carmin: stain half an hour; differentiate in acid alcohol; dehydrate in alcohol; pass through xylol; mount in balsam.

(3) Iron hematoxylin (Heidenhain) the smears, after being passed from 70 per cent alcohol into distilled water, are placed for 4 to 12 hours into a 3½ per cent aqueous solution of sulphate of iron and ammonia (violet salt); rinse carefully in distilled water and then place for 6 to 12 hours in an old hematoxylin solution (hematoxylin 1 gram, absolute alcohol 10 c.c., distilled water 90, in brown bottle); rinse again carefully in water and place back into the iron alum solution, where the preparation is usually very quickly differentiated. The right point of differentiation is determined under the microscope. The chromatin of the nuclei should be deep black; the protoplasm light grayish; wash thoroughly; rinse again in tap-water; pass through the various alcohols into xylol; mount in balsam.

(4) Giemsa Stain: "Moist:" After bichloride fixation, place the smears for 5 or 10 minutes into a solution of iodine (potassium iodide 2 grams, distilled water 100 c.c., Lugol solution 3 c.c.); rinse in water and immediately afterwards place for 10 minutes in a 5 per cent solution of sodium thiosulphate, until the specimen is entirely decolorized; then wash for 5 minutes in running water; stain in diluted Giemsa solution (1 drop to 2 c.c. of distilled water) for 6 to 12 hours, or even longer (with concentrated solution 1 drop to 1 c.c., stain 1 to 2 hours); rinse in distilled water, then pass through the following solutions:

(a) Acetone 95 c.c., xylol 5 c.c.

(b) Acetone 70 c.c., xylol 30 c.c.

(c) Acetone 50 c.c., xylol 50 c.c.

(d) Xylol, pure.

Mount in cedar oil or in neutral balsam. The degree of differentiation depends on the time the specimen has remained in (a), (b), and (c).

(5) Giemsa Stain: "Dry:" Fix the air-dried smears for 10 minutes in absolute or methyl alcohol, then stain in diluted Giemsa solution (1 drop to 1 c.c. of distilled water) for 20 to 30 minutes, occasionally longer; rinse in water under the spigot, dry, and when on slides, examine directly; when on coverslips, mount in paraffin oil or cedar oil, and examine. Dry, unmounted slides can be kept indefinitely without losing the color, by removing the cedar oil after each examination. Place the slides in a dish with xylol and let it afterwards evaporate. Do not wipe the slides with paper!

Make as many drawings as possible from the specimens examined as the morphological conditions are better impressed on memory and it

teaches the student to observe more closely. Drawings are very instructive when different colored pencils are used for making the various parts of the cells; for example, nuclei and their derivatives.

Exercise 45. *Ameba* (a).—

(a) Non Pathogenic.

Inasmuch as the stools of animals and man (in which we search for parasitic ameba) frequently contain the cysts of free, living amebae belonging to the group of "*Limax*" it is advisable to study these protozoa first, to avoid, later on, the possibilities of confusing the same with the true parasites.

All these amebae can very readily be cultivated: Place fresh hay in a glass; cover it with water and let it stand at a temperature of 25 degrees. A similar glass is prepared by placing together with the hay a small amount of soil, and incubating as mentioned above.

Exercise 46. *Sarcosporidia*.—

Examine sections which contain the sarcocystis tentella of a sheep. The sections are stained with iron hematoxylin. If fresh material is available, tease the Miescher's tube and prepare smears and stain with hematoxylin or eosin or Giemsa.

Study the development of the sporoblasts and, later on, the development of the single-shaped spores.

Second Day

Exercise 45. *Ameba* (b).—

In the fine pellicle on the surface of the hay infusion, small amebae can usually be found. Make hanging-drop preparations in saline; smear material on coverslips or slides, and stain "moist," as mentioned above, either with hematoxylin—particularly by Heidenhain's method—and before passing it through the alcohols, stain for a short time with eosin or light green. In such preparations very distinct contrasts can be obtained.

Cultivation.—Distribute a few loopfuls of the pellicle, from the hay infusion, in sterile saline solution and smear a small amount of this suspension on agar plates which have the following composition:

Agar 0.5; tap-water 90; ordinary alkaline broth 10 c.c. After two or three days when kept at ordinary room temperature or 25° C, the ameba, together with the bacteria, have developed and have spread over the entire plate.

In a similar manner the half parasitic ameba, which is found in the cloaca of lizards, can be cultivated and studied.

In all these amebae, note particularly the size, the structure of the entoplasm and ectoplasm and the change in the nuclei. Should multi-

plication forms be present, note the changes which occur in the nuclei. Most of these amebas become encysted after 48 hours on agar, and offer therefore an opportunity for the study of the cysts. When transplanted on a new medium, the germination of new vegetative amebae can be seen.

(b) *Parasitic*.—Two harmless amebae, which are parasitic in man, are widely distributed and the material can readily be obtained for study.

1. *Entameba buccalis* is found in the slime on the teeth or the gums, particularly in carious teeth; as a rule, however, it is rare. Examine in unstained preparations, or fixed specimens. The protozoa can be readily distinguished from the leucocytes by their size and the motility of the pseudopodia.

Note particularly the distinct separation of the hyaline ectoplasm from the granular endoplasm which usually shows debris of leucocytes and bacteria. A contractile vacuole is absent. The nucleus, with a thick nuclear membrane and a small caryosome is usually covered by the nutritive material. Reproduction (by simple binary fission) will rarely be observed, nor will there be any stages preceding the encystment.

2. *Entameba Coli* is found in the colon of healthy people and is eliminated by the stools. In certain localities, up to 75 per cent of the people are infected. If possible, fresh material (that is, stools which have been obtained after treatment of the patient with salts) is examined.

For the preparation of specimens, take a loopful of slimy flakes and dilute it, if necessary, with saline. The material is examined one to two hours after collection. Warm the slides before making this preparation. The amebae are sluggishly motile and are seen easily.

If numerous amebae are seen, make some very thin smears and fix "moist" in bichloride, passing the specimens through the alcohol as mentioned above and stain with iron-hematoxylin or with Delafield.

They can be distinguished from the *entameba buccalis* on account of their size which varies 8 to 70 mic. They differ from the pathogenic amebae on account of the fact that the ecto- and endo-plasma are not distinctly separated when the ameba is at rest. Only during motion is the ectoplasma distinctly seen in the pseudopodia. The vesicular nucleus is rich in chromatin, which a thick membrane and a large caryosome. Of the complicated life history, only a few single stages are perhaps found. For their correct interpretation use the schematic drawings which will be given in the lectures. The cysts are very characteristic, containing eight nuclei.

3. *Entameba muris*.—If no human amebae are available, examine for comparison the intestinal amebae of mice, which are found in about 50 per cent of the animals. When the feces of a mouse contain typical cysts with eight nuclei, then kill the animal and make preparations as usual from the caecum and colon.

The *entameba muris* is smaller than the *entameba coli*. The nucleus is less rich in chromatin, otherwise the two *entamebae* correspond fairly well.

Third Day

(c) Pathogenic.—*Entameba dysenteriae* (tetragena, var *histolytica*).

For the study of the ameba causing the so-called endemic dysentery, use slides which are already fixed and preserved in 70 per cent alcohol. Stain the same with iron hematoxylin as usual. Stain, with iron hematoxylin, sections of the intestinal wall of a patient who has died from amebic dysentery.

Entameba tetragena is the only pathogenic ameba known. It cannot be cultivated. The size varies between 8 to 60 micra. In the living specimen a distinct separation in ecto- and endo-plasm is characteristic. In stained preparations these differences are not noted. The entoplasm contains granules, bacteria and red blood cells. A contractile vacuole is absent.

The nucleus is distinct, spherical, and has a dense double membrane.

The ectonucleus is well developed and rich in chromatin, which is deposited in granules or clumps. In the center is a large or small caryosome in which, by differentiation, a centriol can be seen. Surrounding the caryosome there is always a light zone. Cyclic changes are very characteristic for the *entameba tetragena* nucleus. The reproduction takes place by simple fission.

Cyst formation is rarely seen. The cysts contain not more than four nuclei and usually large clumps of what are thought to be chromidial bodies. *Entameba histolytica* represent the degenerated *entameba tetragena*.

From the above statements it is apparent that the structure of the nuclei of the various forms of ameba is particularly important, and the student should pay special attention to the fine structure of the latter.

Fourth Day

The Flagellates.—

(a) Intestinal Flagellates: Many of the representatives of this group are, for the most part, scavengers in the intestines. They are frequently found accidentally in the secretions of man and their main form should, therefore, be known.

1. *Endo-Lacertae* is selected for the study of the group of proto-mondina. This parasite is found in the cloaca of lizards. Preparations are made by pressing a small amount of feces from the anus of the lizard, diluting with a small amount of saline and then preparing in a drop of very diluted gelatin (to reduce the motility of the flagellates) a hanging-drop or simple coverslip preparations.

For permanent mounts coverslip smears are fixed in bichloride and stained with hematoxylin.

The organism possesses a lancet-shaped body and two flagella: one directed forwards, the other backwards, acting as a trailing flagellum.

In stained preparations, note particularly the basal granules of the two flagella and the rhizoplast. For the mode of multiplication consult text books.

2. *Trichomonas lacertae*, *vaginalis*, or *intestinalis*: The latter representatives of the group of the "polymastigina" are found as harmless inhabitants of the intestinal tract. They are seen occasionally in diarrhoeic stools or in vaginal secretions.

Make preparations as stated above. Examine stained and unstained. The body of the protozoan is ear-shaped, with four flagella which are united to the body by an undulating membrane. The basal granule is very distinct. The cytostome is small and sickle shaped. The nucleus is oval, very rich in chromatin with a small caryosom.

3. *Lambliæ*: This parasite is found in the small intestines of man where it attaches itself to the epithelium. It is characterized by a completely bi-lateral, symmetrical structure. The entire nucleus structure is doubled, with right and left halves. Four pairs of flagella and a sucker-like, depressed area are found on the ventral surface. Each flagellum has its own basal granule; the granules are connected by axostyles. The preparations for the study of the parasite are either made from the intestines of mice or rabbits, where the lamblæ are always attached to the mucous membrane. In the contents of the intestines, typical cysts can occasionally be found.

(b) *Hemoflagellates and Allied Forms (Bi-nucleata)*:

1. *Trypanosoma lewisi* is found in the blood of infected rats, usually in large numbers. Take from the tip of the tail a drop of blood, examine it unstained by placing it on a slide, covering it with a coverslip and, without pressing it, surround it with vaseline or beeswax. Make also some very thin blood smears on slides; inasmuch as the blood protozoa withstand drying fairly well the film is air dried and stained by Giemsa. For diagnostic purposes these dried blood films are very suitable.

Fix "moist" blood smears in bichlorid and stain by the moist Giemsa method as described above.

The size of the trypanosome varies. The posterior extremity tapers abruptly. Note the undulating membrane with the bright red stained (Giemsa) flagellum which arises from a centriol which is in connection with the kineto-nucleus.

In the unstained preparation note the movements of the trypanosome (traveling and wiggling movements) which are due to the undulating membrane.

The protoplasm is, in unstained preparations, bright green, in stained, bluish, with a few granules.

The trophonucleus is at the border of the first and second and third body. It is oval, of a fine alveolar structure. The multiplication takes place by simple fission or multiple continuous fission.

Occasionally, sexually differentiated forms can be seen. Their further development may be studied in the rat flea (*hematopinus spinulosus*). Demonstrations will be given of the parasites found in the mid-gut of the flea.

INFECTION AND IMMUNITY

The Wassermann Reaction.—This reaction, introduced in 1906 by Wassermann, Neisser and Bruck, is an adaption and modification of the Bordet-Gengou reaction of alexin fixation, the test for antigens and antibodies. It was at first thought to be simply another case of specific fixation between *Tr. pallida* and the serum of a syphilitic (antibody). It was later shown that the "antigenic" solution might be replaced by several lipoidal substances, such as lecithin and alcoholic extracts of various normal organs of man and animals. In other words it differs from the original Bordet-Gengou reaction in that the antigen is not strictly specific. The reaction occurs between a lipoid and a lipoidophilic substance in the serum of syphilitics. A few other sera may at times give the reaction (e.g. from such diseases as scarlet fever, malaria, etc.). It is, however, highly indicative of syphilis in the first and second stages of the disease.

In all respects the reaction is carried out as is the fixation reaction. Through the courtesy of Dr. Sawyer, materials for the test will be provided from the State Hygienic Laboratory where tests are made for physicians from blood or cerebro-spinal fluids of suspected syphilitics. With known materials supplied from this source, the reaction will be carried out by two students and demonstrated to the class. The greatest uniformity of method is necessary to obtain results of diagnostic value. The details of technic may be found in general treatises and monographs on the reaction, particularly:

Noguchi: Serum diagnosis of syphilis; Lippincott.

McIntosh & Fields: Syphilis; Longmans, Green & Co. 1911.

TABLE GIVING THE SALIENT CHARACTERS OF THE MORE IMPORTANT MAMMALIAN TRYPANOSOMES

Group	Sub-Group	Trypanosome	Date and Designators	Synonyms	Motility	Morphology		Nucleus	Cytoplasm	Quantitative Characters				Pathogenicity	Development in insect host when known	Where first found	Remarks	
						Flagellum	Blepharoplast			Length Min.	Breadth Max.	in Microns Min.	Max.					
I	A	1	T. brucei	1899 Plimmer & Bradford			Always free				18	34			Acute for all laboratory animals	Unknown	Zululand	
		2	T. evansi	1885 Steel	{ T. soudanese (1907) T. venezuelense (1910) }		Always free				18	34	1-5	2	Acute for all laboratory animals	Unknown	India	
		3	T. hippicum	1910 Darling			Always free				18	28	1-5	3	Acute for all laboratory animals	Unknown	Central America	
	B	4	T. equiperdum	1901 Doflein			Always free				16	35			Acute for all laboratory animals	Transmitted by coitus	North Africa	{ Causes characteristic "plaques."
	C	5	T. equinum	1901 Voges			Always free	Absent			Av.	23		1.5	Acute for all laboratory animals	Unknown	South America	
	D	6	T. vivax	1905 Ziemann	T. cazalboui (1906)	Very rapid, transitory	Always free			Club shaped	16	31	2	3	Equidae and ruminants only	{ Limited to proboscis in glossina }	Cameroon	
		7	T. caprae	1910 Kleine		Very rapid, transitory	Always free			{ Heavier build than Vivax }	18	32	1-75	4.25	Equidae and ruminants only	" "	German East Africa	
		8	T. uniforme	1911 Bruce			Always free				12	19	1-5	2-5	Equidae and ruminants only	" "	Uganda	
	E	9	T. transvaaliense	1903 Laveran			Always free	Near nucleus			18	50	4	6	Slight to Bovidae only	Unknown	Transvaal	
		10	T. theileri	1902 Laveran			Always free				25	70	2	5	Slight to Bovidae only	{ Possibly hippobosca, details unknown }	Transvaal	
	F	11	T. ingense	1909 Bruce			Always free	Near nucleus		Myonemes	72	122	7	10	Unknown	Unknown	Uganda	
		12	T. tragelaphi	1913 Kinghorn & Yorke			Always free	{ Large ovoid, near nucleus }		Myonemes	52	72	5	8-5	Unknown	Unknown	N. E. Rhodesia	
	G	13	T. lewisi	1881 Kent		Very rapid, transitory	Always free	{ Rod shaped, transverse }	{ Junction of ant. & middle thirds }		Av.	24			Rats only infected	{ Intestines of rat, fleas, and lice }	(World wide)	
	H	14	T. cruzi	1909 Chagas		Tumbling movement	Always free				Av.	20			Slight for laboratory animals		Brazil	{ Dividing forms not seen in blood? }
II	I	15	T. congolense	1904 Broden	{ T. dimorphon (1904) T. pecorum (1910) T. confusum (1909) }		{ Always free Never free Never free }			8	19	Av.	1-5	Acute for all laboratory animals	{ Gut and proboscis of Glossina }	Congo	{ Probably these trypanosomes are identical. }	
		16	T. nanum	1905 Laveran		Never free				10	16	Av.	1-5	Equidae and ruminants only?	" "	Soudan		
	J	17	T. montgomeryi	1909 Laveran			Never free			{ Heavier build than congolese }	10	20	Av.	3.3	Slight for laboratory animals	Unknown	N. E. Rhodesia	
	K	18	T. simiae	1912 Bruce	T. ignotum (1912)		Never free	{ Frequently projects as lateral excresence }			12	23			Acute for monkeys and pigs only	{ Proboscis of Glossina morsitans }	Nyasaland	
III	L	19	T. gambiense	1902 Dutton			{ Sometimes free; sometimes not }			13	39			Acute for laboratory animals	{ Gut and salivary glands of glossina }	Gambia		
		20	T. multiforme	1912 Kinghorn & Yorke			"				10	33			Slight for laboratory animals	Unknown	N. E. Rhodesia	
		21	T. nigeriense	1913 Macfie			"				8	37			Slight for laboratory animals	Unknown	Nigeria	
	M	22	T. pecaudi	1907 Laveran			"		{ Posterior Nuclear forms }		14	35			Acute for laboratory animals	{ Gut and proboscis of Glossina }	Senegal	{ Roubaud and Rouet's observations. }
		23	T. rhodesiense	1910 Stephens & Fantham			"		"		12	39			Acute for laboratory animals	{ Gut and salivary glands of glossina }	N. E. Rhodesia	
		24	T. ugandae	1913 Stephens & Blacklock	{ T. brucei (Uganda) (1909) }		"		"		13	38			Acute for laboratory animals	" "	Uganda	
		25	T. equi	1913 Blacklock & Yorke			"		"		14	36			Acute for laboratory animals	Transmitted by coitus	North Africa	

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The reaction consists in adding a lipoidal antigenic solution to the serum to be tested (previously heated to 56 degrees), adding to the mixture a known number of hemolytic doses (e.g. 3) of fresh guinea pig alexin (complement) allowing contact at 37 degrees for one hour and then adding a hemolytic system consisting of 1 c.c. of a 2 per cent or 5 per cent suspension of washed sheep red blood corpuscles plus three minimal doses of hemolytic serum (S. rabbit-anti-sheep, 56 degrees). The occurrence of hemolysis means that the suspected patient's serum is "negative," complete absence of hemolysis that the serum is "positive," that is, that the patient is syphilitic.

The titers of alexin and of the hemolytic serum must be tested in connection with each series of tests. The antigen must be frequently tested. Various controls are necessary, including the carrying through of known "positive" and of known "negative" serum.

The exact protocol of the experiment will be given in connection with the actual materials used in the test.

HISTOPATHOLOGY AND MORBID ANATOMY

First Day

Tumors.—A tumor is an overgrowth of tissue occurring without known cause. It is localized at first, is parasitic, and serves no useful purpose so far as known. It may be composed of a single type of tissue or of tissues representing all three embryonic layers. Tumors are usually single, but may occur double in paired organs as ovaries or kidneys, or they may be multiple as leiomyomata of the uterus and neurofibromata. Tumors may be definitely encapsulated, communicating with surrounding tissue only by a pedicle containing nutritional vessels; such tumors are benign. Encapsulation may be entirely wanting; in the latter case the growth invades the surrounding tissue and frequently its cells reach lymph or blood channels and may be carried to distant parts where they become implanted and continue to grow. Such filtration and the formation of transplants or metastases constitute some of the features of malignancy—autonomy—vitality—function.

Benign Tumors of Connective Tissue Type:

Fibroma, myxoma.

Second Day

Tumors of connective tissue origin.

Lipoma, Leiomyoma, Chondroma, Osteoma.

Malignant tumors of connective tissue type.

Fibro-sarcomata, spindle cell and round cell sarcomata.

Third Day

Cell types found in tumors.

Rapidly growing tumors as contrasted with slow growing ones.

Mitosis—normal, pluripolar, etc. Hyper & hypo-chromatosis.

Variation in cytoplasm—Cytoplasmic degenerations.

Giant cell formation—of foreign body type and giant cells of the tumor proper.

Osteosarcoma.

Osteoid sarcoma.

Giant celled sarcoma, epulis.

Fourth Day

Telangiectases.

Angioma of liver.

Hemangio-endothelioma.

Lymphangio-endothelioma.

Perithelioma.

Dural endothelioma.

Lymphosarcoma.

Malignant lymphoma.

Melano-sarcoma.

FIFTEENTH WEEK

GENERAL OUTLINE

Protozoology.—Hemoflagellates (continued); *Trypanosoma brucei*; *Leishmania*, etc.

Hemosporidia; protosoma, malaria, piroplasma.

Coccidia. Ciliata.

Infection and Immunity.—Second section visits the Cutter Laboratory.

Lectures on Immunity to protozoa; diseases of unknown etiology and cancer research.

Experimental tumors in animals.

Histopathology and Morbid Anatomy.—Tumors (continued).

PROTOZOOLOGY

First Day

Hemoflagellates.—

2. *Trypanosoma brucei*: Make preparations from an infected guinea pig (collect the blood from the ear) or rat in the manner described above, stain as usual.

Morphologically, this form differs from *T. lewisi* by the rounded, posterior end; the flagellum is longer; the trophonucleus is in the middle of the body.

3. *Trypanosoma equiperdum*. Make preparations from an infected rat, as usual. This trypanosoma differs from the above mentioned *T. brucei* on account of its size and the length of the free flagellum.

4. *Schizotrypanum cruzi*: Make preparations from the blood of an infected guinea pig and at the same time impression preparations from the lung, liver, spleen, myocardium, etc., and stain "moist" by the Giemsa method. For comparison, examine also sections of these organs. This particular trypanosome undergoes two different types of multiplication in the internal organs, particularly in the capillaries of the lungs. One type shows multiplication into forms which resemble merozoites of the malaria parasite, which later on, penetrate the blood corpuscles and pass into the general circulation. The second type is entirely intracellular and the appearance and structure of the parasite is similar to *Leishmania*.

5. Examine smears of various pathogenic trypanosomes, e.g., *T. gambiense* and *T. dimorphon*, etc.

6. If possible, examine cultures of *Trypanosoma Americanum* and *brucei*. Some of the trypanosomes can be readily cultivated on blood agar, prepared according to Novy.

7. *Leishmania*.—The parasites causing the disease “Kala-azar” and “*Leishmania infantum*” are examined in smears and sections of the liver spleen, together with preparations from cultures.

The parasites in the cells of the liver are of uniform size, multiplying by fission. Each parasite possesses two distinct nuclear bodies. The cells which harbor the parasites are mainly leucocytes and endothelial cells.

In artificial cultures on blood agar the parasite develops into a typical leptomonad form. The relatively large, rounded forms are pear-shaped and a flagellum is developed at the blunt end of the body.

Second Day

(c) Hemosporidia (part of Bi-nucleata).

1. *Hemoproteus noctuae* or *colombae*. In the blood of owls or pigeons an organism is found within the nucleated corpusele which grows around the nucleus into a halter-like form (halteridium). Make blood smears as usual, from the killed pigeon; make smears of the lungs and stain by Giemsa. In the smear preparations the intracellular parasites in leucocytes or endothelial cells can be easily recognized, on account of the arrangement of the large number of nuclei, which, when breaking up, resemble the Merozoites of the malarial parasite. In the blood corpuseles, the developing forms are easily detected with dry lenses. The student will study the charts on hand to explain the life cycle.

2. *Proteosoma precox*. This parasite is the cause of bird malaria and is used here to illustrate the life history of the hemameba. The development is the same as that described for malaria. The microgamete formation can very readily be followed in hanging drop preparations. Smears are made from one of the blood vessels of the wing of the bird.

3. Examine some smears containing “leucocytetozoon” of birds.

Third Day

(c) Hemosporidia (continued).

4. Malaria. Dried blood smears of the various malarial parasites (*Plasmodium vivax*, *malariae*, *immaculatum*) will be given to the student for staining and study. The life cycle of this parasite is well known and a full description is found in every textbook.

5. Piroplasmas. The student will examine several blood smears already stained, showing *piroplasma canis*, *P. bigeminum* and *P. parva*. The life cycles of these protozoa will be discussed in the lecture. General discussion of the anatomy, economics of the transmitters (mosquitoes, ticks, etc.).

Fourth Day**(d) Coccidia.**

These parasites are of intracellular habitat during the trophic phase and are very frequently found in the small intestines and the liver of rabbits.

The student examines the intestinal contents or the feces of several rabbits. If the typical oval-shaped cysts are found sections are made from the duodenum; stained and unstained preparations are made from lesions of the liver, and also, for comparison, sections already stained with hematoxylin-eosin are examined.

For the staining of the cysts use diluted Delafield hematoxylin and stain for 24 hours.

The life cycles and the characteristic development of the parasites in the cell is fully described in text books to which the student is referred.

(e) *Ciliata*.—The ciliates are in many respects the highest differentiated forms of protozoa. The body is covered by cilia which are arranged in many different ways. The only important parasitic form is:

Balantidium coli: This entozoic form of polytricha is frequently found in the intestines of hogs. Similar forms occasionally cause dysentery in man. Make hanging drop preparations in gelatin solution. Fix very thin smears in bichloride, stain with borax carmine. The oval shaped parasites have a short peristoma, two contractile vacuoles, and a kidney shaped macronucleus; the micro-nucleus lies in its angle.

Multiplication takes place by fission and conjugation. Cysts are also formed.

INFECTION AND IMMUNITY

Experimental Tumors in Animals.—Transplantable tumors in rats or mice will be demonstrated, sections from them shown and some of the problems of cancer research explained.

HISTOPATHOLOGY AND MORBID ANATOMY**First Day**

Gliomata.

Neurocytomata.

Epithelial tumors.

Types of growth; stroma of.

Papilloma.

Adenoma and malignant adenoma.

Carcinoma.

Epidermoid.

Non-cornifying.

Carcinoma of cervix.

Second Day

Epithelial tumors of breast.
Adeno fibromata.
Intercanilicular adenofibroma.
 Carcinoma of breast.
 Adeno-carcinoma.
 Medullary carcinoma.
 Scirrhus carcinoma.
Paget's disease.

Third Day

Carcinomata of gastro-intestinal tract.
Carcinoma of stomach; Adeno-carcinoma; Medullary; Scirrhus;
 Colloid.
Carcinoma of intestines and appendix.
Carcinoma of liver.
Carcinoma of pancreas.

Fourth Day

Epithelial tumors of the genito-urinary tract and adrenals.
Papilloma of bladder.
Papilloma of Penis.
Adeno-cystoma of ovary.
Papillary adeno-cystoma of ovary.
Carcinoma of kidneys; bladder, prostate; penis, adrenals.

SIXTEENTH WEEK

Protozoology.—

Filaria, chlamydozoa; rabies.

Study and identification of unknown micro-organisms.

Histopathology and Morbid Anatomy.—

Tumors (conclude).

Pathology of nervous system.

PROTOZOOLOGY

First Day

Filariasis.—The student examines blood smears which contain the micro-filaria immitis of the dog.

Chlamydozoa.—Various sections are demonstrated showing Guarneri bodies, vaccine bodies, Mallory's scarlet fever bodies, inclusions in epithelioma contagiosum, etc.

Second Day

Rabies.—Negri bodies are characteristic of rabies and are particularly found in Ammon's horn and the cerebellum. For their demonstration, smears, impression preparation and sections of brain substances are made. If the examination for Negri bodies is negative, the gasserian ganglia can be examined for typical local lesions. The method applied for the detection of Negri bodies is as follows: From the fresh brain tissue of the hippocampus a small piece is removed, and, by gentle pressure, this piece of tissue is pressed on the slide (impression preparation) or a small piece is moved over a slide (smear). The smears are then placed in absolute alcohol from two to five minutes, thereupon the alcohol is allowed to evaporate and the smears stained. The best stain to be used for such work is the Van Gieson stain, as follows:

Loeffler's alkaline methylene blue	5 c.c.
Saturated alcoholic solution of fuchsin.....	3-0 drops
Distilled water	20 c.c.

The mixture is poured over the slide and heated over a Bunsen burner several times, until steam rises. The stain is permitted to remain upon the smear 30 to 120 seconds. It is then washed in running water and if the color of the smear is *blue* where the brain tissue is most thick, and *red* where the smear is thin, the slide is placed between filter paper and dried. As soon as the preparation is dried, with a low power lens a

search is made for large nerve cells. These cells are stained deep blue when properly done. The intensity of the staining of the fuchsin will frequently, in the hands of the beginner, be increased at the expense of the blue of the Loeffler's alkaline methylene blue. When properly stained, it is examined with the oil immersion lens. Negri bodies are maroon red and the inside structures are bluish black. In searching a smear for Negri bodies, only those bodies within the nerve cells should be considered. Sections of the hippocampus showing Negri bodies particularly distinctly, will be distributed. They have been stained according to the method of Lentz (see text books).

Sections of the gasserian ganglion will be given, for examination, by the instructor. The characteristic lesions show partial or complete destruction of the ganglion cells, their place being occupied by cells of the endothelial type. The foci consist almost entirely of such cells, but lymphocytes, connective tissue cells and leucocytes may be seen. It is sometimes necessary to examine a number of sections before finding the change.

Third and Fourth Days

Examination for an unknown microorganism.—When material is received at a laboratory for examination the question primarily to be decided is whether only one particular microorganism (for example: in the stools, *B. typhosus* or *Vibrio cholerae*, or diphtheria bacilli in a swab, or, in the sputum, the tubercle bacillus) is of interest, or whether the exact scope of the investigation is not given. In the first case, definite rules are followed, and in the second instance no scheme is at hand. The examination is not a technique, but an art, and the trained bacteriologist will see many possibilities which are entirely obscure to the beginner, and the latter will overlook many methods which seem essential to the trained eye. The usual outline which may aid is given below:

1. Macroscopic examination of the material: consistency, color, odor.
2. Microscopic preparations:
 - (a) Hanging drop;
 - (b) Stained preparations (Gram, for tubercle bacilli, and Giemsa).

This step must always be undertaken, otherwise culture media may be used on which the organisms will not grow. For example: gonococci or meningococci do not grow on ordinary agar. All slides should be labelled and kept for comparison.

3. Examination by means of cultures:

If a well known type of organism is found, proceed in the manner described in the text-books (for staphylococci, agar and gelatin plates; for diphtheria-like rods, Loeffler's serum; for very fine rods, blood media; for vibrios, peptone solutions); if the type is doubtful, as many methods as possible are applied.

Agar and glycerin agar plates are poured and are also inoculated by smearing the material on the surface. If slow growth is to be expected, also inoculate slanted agar tubes to prevent the media from drying, bouillon is used (often the only method by which a few living bacteria can be isolated and grown). For certain bacteria, serum, ascites and blood media are used. For special purposes colored media: Conrad-Drigalski, etc., are taken. Never fail to inoculate one tube of glucose agar as a "deep tube." When anaerobes are suspected from the odor, inoculate brain emulsion with large quantities of material, deep agar and gelatin tubes, with and without glucose or indigo sulphate, are also to be used.

4. Primary animal inoculation: Select, if possible, the animal from which the material was collected for inoculation, or use rabbits, guinea pigs, mice, rats, and inject subcutaneously, or intraperitoneally.

The rest of the material is kept in the ice chest (anaerobic material, dry or moist).

The following day the cultures are examined microscopically, with the hand magnifier and at 60 times' enlargement. Note if the suspected points are *really colonies* (coagulated serum will also be liquefied by pus alone) and note also the *number* of different types of colonies. They are marked and examined by different staining methods. The size and motility vary according to the temperature of cultivation; differences also exist depending upon whether the material has been collected from the top of the agar slant or near the water of condensation. When differences exist in the staining reactions (for example: by Gram), in comparison with the original material, such observations should be considered with great care, as beginners may have overlooked some important part of the method, etc. If motility is present, stain for flagella. In searching for spores, make hanging drop preparations and test the resistance against heat by exposing the culture for a few minutes to a temperature of 80° C, and then make a subculture. Spore formation is best seen on potatoes. If the first finding is negative, repeat the examination after two or three days.

For differential diagnostic purposes, inoculate different media; gelatin, neutral red agar, malachite-green agar, agar with different carbohydrates, etc.

Test whether the microorganism gives serum reactions with the serum of the patient or immunized animals in appropriate instances.

With the pure culture, inoculate animals. If the inoculation is negative with a small quantity, inoculate large amounts by the different methods explained (also in the anterior eye chamber and on the scarified skin). When the animals become diseased, examine whether the bacillus produces a toxin, or if the killed (heat or chloroform) bacteria are toxic.

The microorganisms found are identified by consulting reference books (Neumann and Lehmann; Chester's Determinative Bacteriology and Baumgarten's Jahresbericht). (This examination will be continued in the following week).

MORBID ANATOMY AND HISTOPATHOLOGY

First Day

Adamantinoma. Hypophysial tumors—Pineal gland tumors, Thyroid—Carotid gland lining.

Epidermoid cysts—dermoid cysts —Cholesteratomata.

Mixed tumors.

Hydatid mole and chorio-epithelioma.

Second Day

General review of tumors.

Theories of tumor etiology.

Cohnhei's theory of congenital misplacement.

Ribbert's theory of cell displacement and diminution of normal relations of tissue tension.

Parasitic theories. Filterable viruses and tumors.

Similarities and dissimilarities between embryonic and tumor cells.

Methods of growth of tumor cells—tissue tension.

Growth and vegetative activities.

Tissue injury and new growths.

Criteria of malignancy.

Third and Fourth Days

Pathology of the Nervous System (Neuropathology).

Neuritis; Herpes zoster.

Types of reaction of the histological elements of central nervous system—the nerve cells (axonal alteration, acute alteration, necrosis, calcification); the glia (hypertrophy, pigmentation); myelinic degenerations—compound granule cells. Vascular alterations in acute infections; in chronic infections. Little diseases and Birth palsies.

Some type diseases of the nervous system.

Some type diseases of the spinal cord.

Anterior poliomyelitis.

Progressive muscular atrophy and amyotrophic lateral sclerosis.

Tabes dorsalis.

Syringomyelia.

Multiple sclerosis.

Huntington's Chorea.

Paralysis agitans.

Amaurotic Idiocy.

Epilepsy.

Arterio-sclerosis.

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